ARTICLE

Incorporation of Oleic Acid and Eicosapentaenoic Acid into Glycerolipids of Cultured Normal Human Fibroblasts

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Confluent skin fibroblasts from normal humans were incubated in serum free medium with up to 100 nmole/mL eicosapentaenoic acid (EPA; bound to albumin in a 4.6:1 ratio) and compared with cells incubated with oleic acid (OA) at similar concentrations. The rate of [^{14}C]OA incorporation into triacylglycerol (TG) (nmol/mg/h) was approximately 5-fold greater than that of [^{14}C]EPA. The mass of TG formed after incubation of fibroblasts with EPA was also significantly lower than that formed with OA (43.2 ± 9.3 *vs.* 59.5 ± 6.6 μg/mg cell protein, respectively, $P = 0.006$). The addition of excess, unlabeled EPA reduced the rate of incorporation of [^{14}C]OA into TG whereas unlabeled OA stimulated incorporation of [^{14}C]EPA into TG. When the cells were preincubated with human serum basic proteins (BP I, II and III), the mass of TG formed (compared to baseline) was significantly higher with the basic proteins whether OA or EPA was studied. Only BP I significantly stimulated the mass of cell phospholipids, an effect that occurred with either OA or EPA in the medium. The results suggest that in cultured normal human fibroblasts, OA is a better substrate for TG synthesis than EPA and that this effect may be accentuated by the presence of the basis proteins.
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Long-chain n-3 polyunsaturated fatty acids have a number of beneficial effects including inhibition of hepatic very low density lipoprotein (VLDL) production (1). Inhibition reflects reduced lipogenesis and enhanced fatty acid oxidation (2). This results in decreased formation of triacylglycerols (TG) (3). In cultured Hep G-2 cells, for example, eicosapentaenoic acid (EPA), the archetype of the omega-3 fatty acids, inhibits TG synthesis as has been shown by reduced [^{3}H]triacylglycerol production from [^{3}H]glycerol (4). In mammalian cells such as fibroblasts, endothelial and intestinal cells, polyunsaturated fatty acids appear to be selectively incorporated into phospholipids (5-7).

In contrast to EPA, monounsaturated fatty acids, such as oleic acid (OA), are readily incorporated into TG. In cultured fibroblasts, OA comprises a substantially greater percentage of the fatty acid pool in total neutral lipid esters (8). The addition of EPA, however, inhibits the stimulation of hepatic TG secretion by OA (1,9). Taken together, these observations suggest that EPA and OA affect TG synthesis differently; EPA inhibits while OA stimulates the process.

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Abbreviations: ASP, acylation stimulatory protein; BP, basic protein; DG, diacylglycerols; EPA, eicosapentaenoic acid; FCS, fetal calf serum; FFA, free fatty acids; GLC, gas-liquid chromatography; MEM, minimum essential medium; MG, monoacylglycerols; OA, oleic acid; PBS, phosphate buffered saline; TG, triacylglycerols; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

Recently, we and others reported a two- to threefold stimulation of [^{14}C]OA incorporation into cellular TG of normal human fibroblasts by a novel human serum basic protein, BP I, also termed acylation stimulatory protein (ASP) (10,11). Two other basic proteins (BP II and BP III) were also described, but their acylation stimulatory activity was less than that of BP I (11).

Our objectives here were to determine (i) whether the differential effects of OA and EPA on fatty acid incorporation into hepatocyte TG were also expressed in cultured normal human fibroblasts, and (ii) whether serum basic proteins affect incorporation of EPA into cellular glycerolipids in a similar manner as previously demonstrated with OA.

MATERIALS AND METHODS

Subjects. Skin biopsies were obtained from the forearms of six nonsmoking, non-obese, normolipidemic volunteers after informed consent. The mean age of the three male and three female donors was 24.2 yrs. The mean levels (mg/dL) of lipids and lipoproteins were: total cholesterol, 142, triglycerides, 43, low density lipoprotein cholesterol, 60, and high density lipoprotein cholesterol, 73 (11).

Cultured fibroblasts. To avoid experimentation with very young (*i.e.*, passage number <3) or senescent (*i.e.*, passage number >12) cells, passage numbers between 4-10 were used; during these passages, mitotic division is active. For each experiment, cells were plated on 35-mm or 60-mm Petri dishes in Eagle's minimal essential medium (MEM), containing 10% fetal calf serum (FCS), 1% nonessential amino acids and 1% penicillin-streptomycin. On day 6, FCS was replaced with serum-free medium, and the cells were incubated overnight prior to the experiment.

Fatty acid supplementation. [^{14}C]Oleic acid (specific activity, 57 mCi/mmol) and [^{14}C]EPA (specific activity, 52.2 mCi/mmol) were obtained from Amersham (Arlington, IL). Nonradiolabeled fatty acids were purchased from Sigma (St. Louis, MO) and pre-tested for purity (>95%) by gas-liquid chromatography (GLC) of the methyl ester. Unlabeled OA (90 mg) or unlabeled EPA (96 mg) were solubilized in 5 N NaOH (100 μL) and ethanol (2 mL). The solvent was evaporated to dryness with N_2, and 150 mM NaCl (10 mL) was then added to a final concentration of 31.9 mM and heated for 5 min at 60°C (12). Bovine serum albumin (24%, wt/vol, fatty acid free) in 150 mM NaCl (12.5 mL) was added and the solution stirred overnight. The solution was then adjusted with 150 mM NaCl to give a final concentration of 12.7 mM sodium oleate or 12.7 mM sodium eicosapentaenoate in 12% albumin (wt/vol). [^{14}C]OA/albumin and [^{14}C]OA/albumin and [^{14}C]EPA/albumin were then prepared to give preparations of 10 mM oleate or 10 mM eicosapentaenoate; specific activity, about 10,000 dpm/nmol, molar ratio of oleate or eicosapentaenoate to albumin (4.6:1) (11).

Free fatty acid (FFA) incorporation experiments. On day 7, dishes were incubated with increasing amounts of

[^{14}C]OA/albumin or [^{14}C]EPA/albumin (final concentration of fatty acid, 10 mM), for 1–2 h at 37°C. At the end of the incubation period, cells were placed on ice, and media removed. Cells were then washed thoroughly with phosphate buffered saline (PBS) and bovine serum albumin. Lipids were extracted with hexane/isopropranol (3:2, vol/vol), and the extract was taken to dryness with N_2. The lipids were then dissolved in hexane and subjected to thin-layer chromatography (TLC) employing hexane/diethyl ether/acetic acid (75:25:1, vol/vol/vol) as solvent. The incorporation of [^{14}C]OA or [^{14}C]EPA into cellular lipids was determined by scintillation counting and expressed as nmol incorporated/mg cell protein/h. In experiments designed to measure incorporation into monoylglycerols (MG) and diacylglycerols (DG), the TLC solvent system was ethylenedichloride/methanol (98:2, vol/vol) (13).

Competition experiments. Cell dishes were incubated with increasing amounts of [^{14}C]OA/albumin or [^{14}C]-EPA/albumin as described above in the absence or presence of unlabeled OA/albumin or unlabeled EPA/albumin (25 μg fatty acid/mL medium). Extraction of cellular lipids was done as described above.

Basic protein experiments. BP I, II and III were isolated from normal human serum as previously described (11). Cells were grown to near confluence, and on day 6 medium was changed from 10% FCS/MEM to delipidized MEM/F12 supplemented overnight with insulin, pantothenic acid and biotin. In fibroblasts grown in serum-deficient medium, the addition of these supplements maximizes cell viability (14). Subsequently, either OA or EPA, and BP I, II or III (6 μg/mL) were added to the media, and the cells incubated for 6 h (11). The medium was removed, cells were washed and lipids extracted.

Mass measurements. The amount of cellular TG was determined using an enzymatic assay (Boehringer Mannheim, Indianapolis, IN). The content of phosphorus in the cell lipid fraction was measured using the method of Bartlett and multiplied by 25 to express the total phospholipid content in mg% (15). Cellular protein content was determined using the method of Lowry *et al.* (16) with serum albumin as standard.

Statistical methods. Tests of significance were made using the paired *t*-test. In some cases, the Wilcoxon Rank Sum was used. The designated level of significance was $P < 0.05$.

RESULTS

The incorporation of OA into cell lipids was compared to that of EPA at increasing concentrations of the fatty acids (nmol/mL) in the culture medium (Fig. 1). The incorporation of [^{14}C]OA (nmol/mg/h) into triacylglycerols was significantly greater than that of EPA at each concentration tested (Fig. 1). In contrast, the pattern of incorporation for [^{14}C]EPA into cell phospholipids was greater than that of [^{14}C]OA, but this difference did not reach statistical significance (Fig. 1). For cellular cholesteryl esters, incorporation of [^{14}C]OA was greater than that of [^{14}C]EPA (Fig. 1).

We next compared the incorporation of [^{14}C]OA *vs.* [^{14}C]EPA into cellular MG, DG (both the 1,2-isomer and the 1,3-isomer), TG and phospholipids (Fig. 2). The incorporation of [^{14}C]OA was significantly greater than that of [^{14}C]EPA into cell 1,3-DG and TG (Fig. 2). No significant differences were observed for the incorporation of these two free fatty acids (FFA) into cell MG. In contrast, the incorporation of [^{14}C]EPA into cell phospholipids was significantly greater than that of OA (Fig. 2). The data confirmed the pattern of greater incorporation of OA into cell TG but higher incorporation of EPA into cell phospholipids.

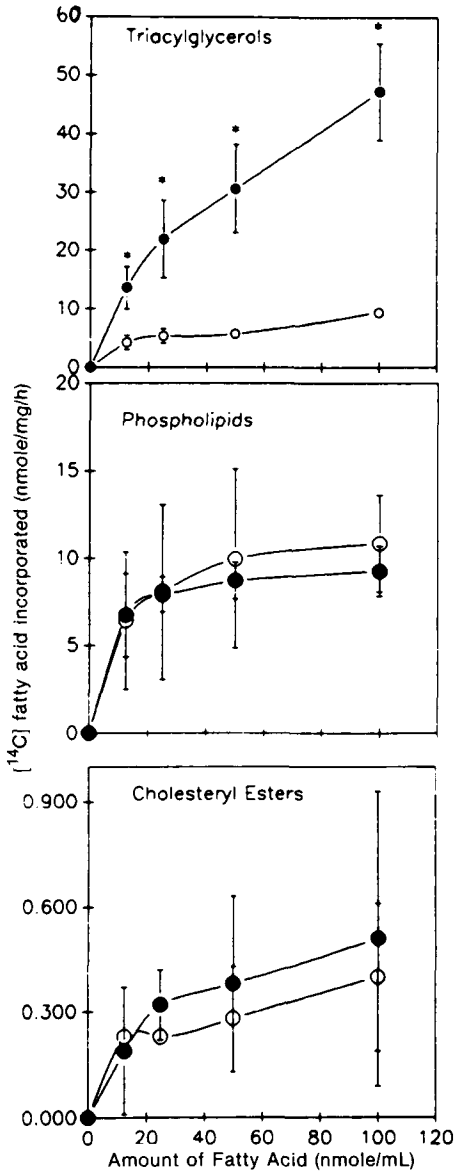

FIG. 1. Incorporation of [^{14}C]oleic acid (OA) and [^{14}C]eicosapentaenoic acid (EPA) into cellular triacylglycerols (top), phospholipids (middle) and cholesteryl esters (bottom) of normal human cultured fibroblasts. Cells were grown as described in Materials and Methods. On day 7, cells were incubated with increasing concentrations of [^{14}C]OA/albumin or [^{14}C]EPA/albumin (10 mM oleate or eicosapentaenoate, specific activity, 10,000 dpm/nmol) for 1 or 2 h at 37°C. Cells were then harvested and the incorporation of [^{14}C]OA (solid circles) and [^{14}C]EPA (open circles) into cell lipids was determined. The results represent the mean (±SD) of duplicate samples from two different cell lines. Those points that were significantly different between [^{14}C]OA and [^{14}C]EPA are designated with an asterisk.

Competition studies of OA and EPA. We next compared the effect of excess unlabeled OA or excess unlabeled EPA

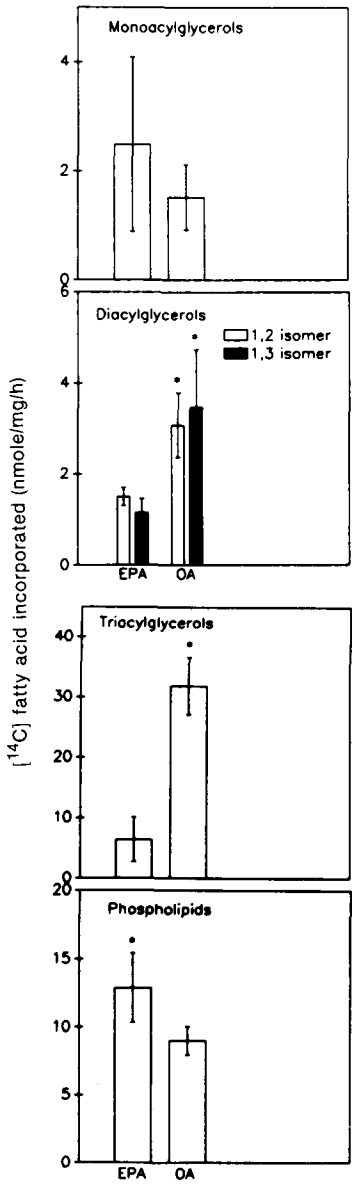

FIG. 2. Incorporation of [^{14}C]oleic acid (OA) and [^{14}C]eicosapentaenoic acid (EPA) into cellular monoacylglycerols, 1,2- and 1,3-diacylglycerols (upper two panels), triacylglycerols and phospholipids (lower two panels) of normal human cultured fibroblasts. Cells were grown as described in Materials and Methods. On day 7, cells were incubated with 50 nmol of either [^{14}C]OA/albumin or [^{14}C]-EPA/albumin (specific activity [^{14}C]OA, 11,128 dpm/nmol, [^{14}C]EPA, 11,765 dpm/nmol) for 1 h at 37°C. Cells were harvested and the incorporation of [^{14}C]OA and [^{14}C]EPA into cellular glycerolipids was determined (see Materials and Methods).

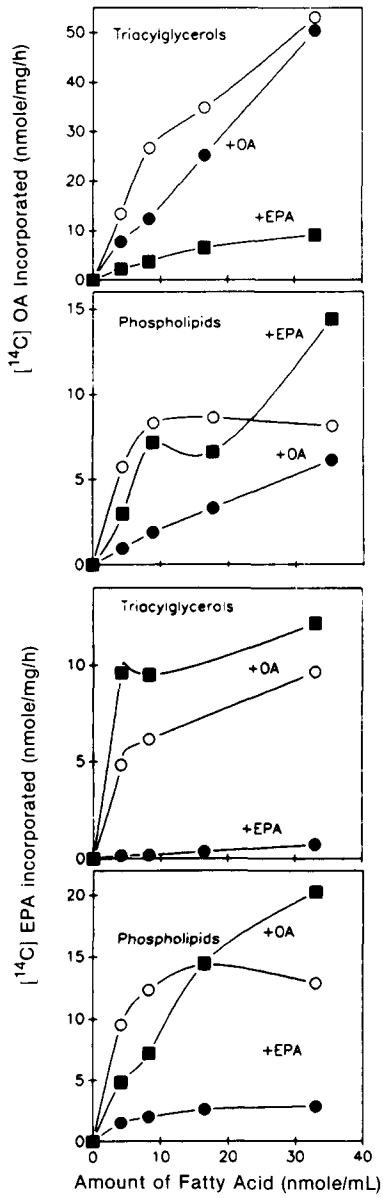

FIG. 3. Incorporation of [^{14}C]oleic acid (OA) (upper two panels) and [^{14}C]eicosapentaenoic acid (EPA) (lower two panels) into triacylglycerols and phospholipids in the absence (○) or presence (● and ■) of 25 μg/mL medium of unlabeled fatty acid. In the upper two panels, the effects of unlabeled OA (●) or EPA (■) on [^{14}C]OA (○) incorporation into triacylglycerols and phospholipids are shown. In the lower two panels, the effects of unlabeled EPA (●) or OA (■) on [^{14}C]EPA (○) incorporation into triacylglycerols and phospholipids are shown. Cells were grown as described in Materials and Methods. On day 7, cells were incubated with [^{14}C]OA/albumin or [^{14}C]EPA/albumin (10 mM oleate or eicosapentaenoate, specific activity, [^{14}C]OA, 11,128 dpm/nmol; [^{14}C]EPA, 10,203 dpm/nmol) for 1 h at 37°C. Extraction of cellular lipids was carried out as described in Materials and Methods.

on the rate of incorporation of either [^{14}C]OA or [^{14}C]EPA into cell TG and phospholipids (Fig. 3). Unlabeled EPA inhibited the incorporation of [^{14}C]OA into cellular TG to a markedly greater extent than unlabeled OA (Fig. 3). In contrast, the incorporation of [^{14}C]EPA into cellular TG was actually stimulated by unlabeled OA; unlabeled EPA markedly depressed the incorporation of [^{14}C]EPA into TG. The incorporation of [^{14}C]OA into cell phospholipids was inhibited by unlabeled OA but was somewhat stimulated by unlabeled EPA, particularly at higher concentration of [^{14}C]OA in the cell culture medium (Fig. 3). Incorporation of [^{14}C]EPA into cell phospholipids was inhibited by unlabeled EPA, while unlabeled OA decreased such incorporation at lower concentrations but increased it somewhat at higher concentrations.

Effect of preincubation with serum basic proteins. We next assessed the effect of normal human serum BP I, II and III on the mass of cellular TG and phospholipids in

TABLE 1

Cellular Mass of Triacylglycerol and Phospholipids (μg/mg cell protein) After Preincubation with Serum Basic Proteins in the Presence of Either Albumin/Oleic Acid (OA) or Albumin/Eicosapentaenoic Acid (EPA)[a]

	OA	*P* value	EPA	*P* value	OA *vs.* EPA *P* value
Triacylglycerol					
Without BP	59.5 ± 6.6[b]		43.2 ± 9.3		0.01
BP I	117.6 ± 30.8	0.007	78.2 ± 8.5	0.0001	0.15
BP II	76.2 ± 15.0	0.07	59.5 ± 10.5	0.02	0.97
BP III	69.3 ± 5.3	0.04	69.9 ± 13.9	0.02	0.13
Phospholipids					
Without BP	335.3 ± 103.4		244.6 ± 31.6		0.10
BP I	628.8 ± 222.7	0.05	556.2 ± 60.7	0.0001	0.90
BP II	440.0 ± 121.9	0.26	265.6 ± 66.5	0.58	0.41
BP III	352.4 ± 90.8	0.83	274.8 ± 98.0	0.48	0.74

[a]Confluent fibroblasts were incubated for 24 h in serum-free medium as described in Materials and Methods. The cells were then switched to medium containing albumin/OA or albumin/EPA with or without BP I, BP II or BP III (6 μg/mL) for 6 h. The cells were then harvested and the TG and phospholipid mass in cells were determined as described in Materials and Methods.

[b]Mean (one SD) in μg/mg cell protein from three normal cell lines studied in duplicate.

normal human fibroblasts, using either OA/albumin or EPA/albumin as the source of FFA in the cell culture medium. BP I, BP II and BP III each increased the cellular mass of TG over baseline; a similar pattern was found when either OA or EPA was used (Table 1). When the cell data on TG mass (OA *vs.* EPA) were compared, there was a significantly higher mass of TG formed at baseline (in the absence of BP I, II or III) with OA than with EPA, consistent with the previous patterns presented in Figures 1 and 2. When OA was compared with EPA in the presence of BP I, II or III, the baseline values were first subtracted from the values following incubation with BP I, II or III. After correction for baseline differences between OA and EPA, there were no significant differences between the effects of BP I, II or III on increasing the cell TG mass, when OA was compared with EPA as the source of fatty acid in the culture medium (Table 1).

Similar analyses were performed to assess the effect of serum basic proteins on the cellular mass of total phospholipids. BP I, but not BP II or BP III, significantly increased the mass of phospholipids compared to baseline, whether OA or EPA was used (Table 1). However, no significant differences were observed at baseline between the cell mass of phospholipids following incubation of the cells with either OA or EPA alone. Correcting for baseline, the extent of the stimulation of phospholipid mass by BP I was not significantly different when OA was compared with EPA (Table 1).

DISCUSSION

The most important observations were (i) that OA was preferentially incorporated into TG while EPA was preferentially incorporated into phospholipids of normal human fibroblasts; (ii) that EPA appeared to inhibit the synthesis of TG from OA, while OA stimulated the formation of TG from EPA; and (iii) that serum BP I, II and III increased the cell mass of TG when either OA or EPA was used (BP I also stimulated phospholipid mass). These observations were made under defined experimental conditions that were selected to produce maximal fatty acid modifications in human cultured fibroblasts (8) and which had previously been found to permit maximum acylation stimulatory activity of the serum (BP) (11).

Our studies with normal human fibroblasts support the findings of most (2-4,9,17,18), but not all (19), previous studies done on hepatocytes, which also showed preferential incorporation of OA into cellular TG, as judged by both mass measurements and by ^{14}C-incorporation into TG. In fibroblasts, we found here that incorporation of EPA into both DG (1,2- and 1,3-isomers) and TG was markedly reduced. Moreover, the presence of excess unlabeled EPA reduced [^{14}C]OA incorporation into TG. This supports previous studies done on hepatocytes where EPA was shown to reduce TG synthesis and to inhibit OA stimulation of TG secretion (1,9,17). However, we did not find any significant differences in the mass of cell phospholipids after incubation with EPA or OA. This was not unexpected, since we used experimental conditions similar to those of Spector *et al.* (8) in which the TG content of cells increased when grown in the presence of added fatty acids, but the phospholipid content remained unchanged.

Our results further suggest that EPA and OA may be metabolized differently in cultured mammalian cells. Previous studies have revealed preferential incorporation of OA over EPA in adipose cells and a preference for EPA over OA in other tissues (*i.e.*, platelet membranes) (20-23). While the mechanism(s) responsible for these differences are now known, it has been shown that activity of the enzymes regulating TG synthesis may be affected by external factors (24). Marsh *et al.* (25) and Wong and Marsh (26) reported a marked reduction in phosphatidate phosphohydrolase activity in rat hepatocytes following a diet rich in EPA, or after infusion of EPA into perfused rat livers. In our studies, EPA incorporation into both DG and TG was markedly reduced, compatible with an effect on phosphatidate phosphohydrolase which hydrolyzes phosphatidic acid to diacylglycerol, rather than an effect of EPA on 1,2-diacylglycerol acyltransferase (17).

We extended our previous studies where we found that BP I, II and III stimulated the incorporation of [^{14}C]OA

into TG of normal fibroblasts by showing here that preincubation of cells with normal human serum BP also increased the mass of TG. This effect was observed whether OA nor EPA was used as a source of fatty acids in the medium (Table 1). While the increase in the mass of TG with various BP was less with EPA than with OA, the differences were not statistically significant after correcting for baseline differences. Thus, we have no evidence for a greater avidity of BP to interact with OA as compared with EPA, or that their cellular effect is more specific for OA than EPA. Finally, we found evidence that BP I may also increase the mass of cell phospholipids. Since neither OA nor EPA alone stimulated phospholipid mass (as expected from the work of Spector *et al.*, ref. 8), this effect of BP I may be a specific one. Further work is indicated to determine whether BP I has any influence on membrane structure and function and what the underlying mechanism(s) may be.

In summary, the data suggest that the metabolic effects of EPA and OA in cultured normal human fibroblasts are different. Human skin fibroblasts from normal and dyslipidemic subjects may provide a useful system to further study glycerolipid metabolism, as we recently demonstrated for hyperapobetalipoproteinemia (27). The serum basic proteins may contribute further to understanding the metabolic processes underlying cellular free fatty acid uptake and utilization in this and other lipid disorders.

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Influence of Dietary Egg and Soybean Phospholipids and Triacylglycerols on Human Serum Lipoproteins

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Human serum lipid and lipoprotein concentrations and compositions were compared in ten healthy middle-aged men consuming phospholipids from egg or from soybean or triacylglycerol mixtures with fatty acid compositions similar to those of the phospholipids. All subjects followed each of the four treatments: egg phospholipids (EP), soybean phospholipids (SP), an oil of fatty acid composition similar to that of EP, and an oil similar in fatty acid composition to SP for six weeks with "wash-out" periods of similar duration between treatment periods. The phospholipids, 15 g/d, and the oils, 12 g/d, which contained approximately equivalent quantities of fatty acids were provided to the subjects in gelatin capsules and were taken before meals. Diet intake was monitored by three-day food records. Serum lipoproteins (L_p) were separated by ultracentrifugation into very low density lipoproteins, low density lipoproteins (LDL), high density lipoproteins $(HDL)_2$ and HDL_3. L_p fractions and whole serum were analyzed for triacylglycerols, cholesterol (CH), phospholipids (PL), and protein. HDL cholesterol was determined in whole serum. Cholesteryl esters were determined in some L_p fractions. Lipid compositions of L_p were expressed in mmol/g protein. Apoprotein B was measured in whole serum and in LDL; apoprotein A-I in whole serum and in HDL_3. In whole serum, CH and PL were significantly lower after the SP compared to EP treatment periods. CH, but not PL, was lower after SPTG compared to EP. CH in HDL_2 was significantly higher after SP compared to SPTG. Also, PL in HDL_2 were significantly higher after SP compared to all other treatments and to baseline. Although human serum lipid responses to dietary phospholipids were generally the same as responses to ingested oils of comparable fatty acid composition, the data suggest the possibility that SP selectively increase HDL_2 cholesterol and phospholipids.
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Although "lecithin," primarily from soybeans, is widely used as a hypocholesterolemic and antiatherogenic agent, experimental tests of its effectiveness are inconsistent. Knuiman *et al.* (1) reviewed the literature to answer the question, Does ingested lecithin affect human serum cholesterol independent of its linoleic acid content? These authors found only four out of 24 studies in which human cholesterolemic responses to soybean lecithin were compared to polyunsaturated oils of similar linoleic acid content. They concluded that lecithin, phosphatidylcholine (PC), had no different effect on serum cholesterol than soybean oil. However, the issue concerning its effect on serum levels of high density lipoprotein cholesterol (HDLC) was not addressed by Knuiman and co-workers (1). HDLC is particularly important because of its inverse relationship to coronary heart disease (CHD) (2). Childs *et al.* (3) found a significant increase in HDLC in response to soybean lecithin compared to an equivalent amount of polyunsaturated fatty esters ingested as corn oil. Total serum cholesterol (TSC) levels were not significantly different between treatment groups. Galli *et al.* (4) administered 10 g/d of purified polyunsaturated phosphatidylcholine to male human volunteers for six weeks. They found no change in TSC but a significant increase in HDLC.

In a previously reported experiment with hypercholesterolemic guinea pigs, we compared cholesterolemic responses to soybean "lecithin" and to egg "lecithin," commercial preparations which were principally PC (5). Since the dietary "lecithins" used in that experiment with guinea pigs were mixtures and not lecithin, the term "phospholipids" will be used hereafter to refer to the polar lipid mixtures. Comparisons to oils of similar fatty acid compositions were not made. Soybean phospholipids resulted in a 50% reduction in TSC while HDLC increased 23%. When the guinea pigs were treated with egg phospholipids, we observed a dramatic increase of 177% in HDLC with no change in TSC. The ratio of TSC to HDLC in hypercholesterolemic guinea pigs that were not treated with phospholipids was 5.76. The groups of animals treated either with soybean phospholipids or with egg phospholipids had TSC/HDLC of 2.26 and 2.24, respectively. The egg phospholipids had a low ratio of polyunsaturated (P) to saturated (S) fatty acid composition (P/S = 0.38) compared to soybean phospholipids with a P/S of 3.5. These data suggested a cholesterolemic response to ingested phospholipids that was not totally dependent upon its fatty acid composition. The results of thisguinea pig experiment were inconclusive because we did not control the fatty acid compositions of the phospholipids nor could we extrapolate the results to responses in humans.

The present study was undertaken to evaluate human serum lipoprotein (L_p) responses to orally administered soybean and egg phospholipids relative to those responses to oils of similar fatty acid composition. In addition to determining serum concentrations of lipids, the lipid composition and the protein content of the major L_p fractions, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL_2) and HDL_3, were determined.

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Abbreviations: apoA-I, apoprotein A-I; apoB, apoprotein B; BHT, butylated hydroxytoluene; CE, cholesteryl ester; CH, cholesterol; CM, chylomicrons; EP, egg phospholipid; EPTG, triacylglycerol mixture with a fatty acid composition similar to EP; GLC, gas-liquid chromatography; HDL, high density lipoproteins; HDLC, high density lipoprotein cholesterol; IDL, intermediate density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; L_p, serum lipoproteins; PBS, phosphate buffered 0.15 M NaCl, pH 7.40; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, serum phospholipids; P/S, ratio (w/w) of polyunsaturated to saturated fatty acids; SP, soybean phospholipids; SPTG, triacylglycerol mixture with a fatty acid composition similar to SP; TG, serum triacylglycerols (triglycerides); TLC, thin-layer chromatography; TSC, total serum cholesterol; UC, unesterified cholesterol; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Subjects. The subjects were 10 normolipidemic men in the age range of 30 to 64 years with an average age (±SD) of 44.4 ± 10.3 y and without metabolic disease or obesity (BMI 25.2 ± 3.0 kg/m^2). None of the men were taking any medication known to alter blood lipids. The project was approved by the Institutional Review Board, Research with Human Subjects, and each subject gave his informed written consent. The subjects were Texas A&M University employees, predominantly scientists, who conscientiously adhered to the experimental protocol.

Test lipids. The test lipids were two phospholipid mixtures and two mixtures of triacylglycerols (triglycerides) with fatty acid compositions similar to those of the respective dietary phospholipids. The phospholipids were "soybean lecithin" (SP) provided by the American Lecithin Company (Atlanta, GA) and "egg lecithin" (EP) provided by the Asahi Chemical Industry Company (Tokyo, Japan). After determining the fatty acid compositions of SP and EP, mixtures of oils were prepared which had fatty acid compositions similar to those of the phospholipids. These triglyceride mixtures are referred to as soybean phospholipid triglycerides (SPTG) and egg phospholipid triglycerides (EPTG). To achieve the appropriate fatty acid compositions in readily absorbable oil mixtures, the following fats (oils) were used: edible beef tallow (compliments of Bunge Edible Oil Corp., Fort Worth, TX) which contained 116 mg cholesterol/100 g tallow (2.70 mmol/L), refined palm oil (compliments of Conway Oil Company, Denison, TX), edible linseed oil (Arista Industries, Darien, CT) and safflower oil (Hain Pure Food Co., Inc., Los Angles, CA). SPTG was 59% safflower oil, 29% palm oil and 12% linseed oil; and EPTG was 65% beef tallow, 31% palm oil, 4% safflower oil and 0.75% cholesterol. Antioxidant [0.01% butylated hydroxytoluene (BHT)] was included in each triglyceride mixture. The lipid and fatty acid compositions of the phospholipids and the triglycerides are shown in Table 1.

Experimental design. The duration of the experiment was divided into seven six-week periods. These included the four test lipid periods of six weeks each: SP, EP, SPTG, and EPTG, separated by six-week periods without supplementation, the "wash-out" periods. Each man followed each test period. In order to minimize bias associated with the order of the test lipids, the subjects were divided into four groups, and each group followed a different order, *i.e.,* Latin square. Initially, there were 12 subjects who were divided into four groups of three. After the first treatment period, two subjects from different groups withdrew from the study for personal reasons. Data resulting from those two subjects were not used.

The four test lipids were contained in opaque, color-coded gelatin capsules (SNAP-FIT capsules size 00, Capsugel, Greenwood, SC) and were taken by each subject as supplements to his habitual diet. The phospholipids were ingested at the level of 15 g/d; and the triglycerides, at the level comparable to the fatty acid content of the phospholipids, 12 g/d. Childs *et al.* (3) had found a significant increase in HDLC in response to 36 g/d SP that was 29% PC. The soybean phospholipid preparation that we used in this study was 69% PC (Table 1). Thus, 15 g SP was approximately equivalent in PC to the preparation used by Childs and co-workers (3) and by Galli *et al.* (4). The daily dose of 30 capsules was taken as 10 capsules before each meal. The subjects returned unused capsules at the end of each treatment period. One subject missed two days during one treatment period. Otherwise, adherence was 99% or better. In order to control for the effect of the capsules themselves on blood lipids and to allow the subjects to become accustomed to taking 30 capsules per day, the men took 30 empty capsules daily for two weeks before the baseline (initial) blood sample was taken.

Although no modification of the habitual diet of these subjects was planned, they were instructed to maintain their initial habitual diet for the duration of the experiment. In order to monitor their habitual diet, the subjects kept three-day diet records (a weekend day and two week days) during each test diet period. The subjects were instructed in the procedure for keeping a food record, and food scales were provided to aid in recording serving sizes.

TABLE 1

Lipid Compositions of Dietary Phospholipids and Oils (%)

Lipid component	Egg phospholipids	Egg phospholipid fatty acids	Soy phospholipids	Soy phospholipid fatty acids
Triglycerides	10.6	100	6.0	100
Phospholipids	73.0	0	68.8	0
Phosphatidylcholine (PC)	75.4		78.3	
Phosphatidylethanolamine	23.4		19.3	
Sphingomyelin	1.2			
LysoPC			2.3	
Fatty acids				
16:0	31.4	32.5	16.3	17.9
16:1n-9	0.3	4.5		
18:0	17.4	8.2	3.3	1.8
18:1n-9	33.4	41.1	11.8	20.2
18:2n-6	14.2	13.7	62.8	52.1
18:3n-3	0.6		5.7	8.1
20:4n-6	3.0			
Polyunsaturated/saturated fatty acid ratio	0.35	0.34	3.5	3.1

These records were analyzed for energy and nutrient contents (Nutripractor 6000, Practocare, Inc., San Diego, CA). The subjects were cautioned to maintain a constant weight (±3 kg) by modifying serving sizes of diet ingredients.

Blood samples were taken at the Texas A&M University Health Center. When the subjects had their initial blood sample taken, measurements of resting blood pressure, body weight and height were also made. Body weight and height were measured with light clothing but without shoes. Blood (15 mL) was sampled at the beginning and end of each test period following a 12- to 14-h fast. The subjects were seated, and the blood samples were taken by venipuncture from an antecubital vein into separator vacutainer tubes containing clot-activating gel. The tubes were placed in wet ice. Within three hours the serum fraction was isolated by centrifugation at 2000 × *g* for 30 min at 4°C.

VLDL, LDL, HDL_2 and HDL_3 were separated from serum by ultracentrifugation and analyzed for lipid composition and protein content. The lipid composition of the unfractionated serum was also determined.

Analytical methods. The phospholipid-class compositions of the SP and EP were analyzed by determining the phosphorus content of the phospholipid (PL) fractions (6) which had been separated by thin-layer chromatography (TLC) (0.5 mm Adsorbosil-Plus, Alltech Associates, Inc., Applied Science Labs, Deerfield, IL) using the solvent system chloroform/methanol/water (65:25:4, vol/vol/vol). The fatty acid compositions of the phospholipids, component oils, oil mixtures and serum PL were determined by gas-liquid chromatography (GLC) of fatty acid methyl esters which were prepared by BF_3-catalyzed transesterification with methanol (7).

L_p were separated from a 4-mL aliquot of serum by ultracentrifugation according to the procedure of Havel *et al.* (8). The density ranges for the L_p fractions are: VLDL, d < 1.006 g/mL; LDL, d = 1.019–1.063 g/mL; HDL_2, d = 1.063–1.125 g/mL; HDL_3, d = 1.125–1.21 g/mL. The intermediate density lipoprotein (IDL) fraction, d = 1.006–1.019 g/mL, was separated, but it was not analyzed in this study. Briefly, each L_p fraction was separated sequentially from serum by flotation using a Ti 50 fixed-angle rotor in an ultracentrifuge (L2-65B, Beckman Instruments, Palo Alto, CA) at 110,000 × *g* for 22 h at 12°C. Density adjustments were made using either a stock salt solution; NaCl + KBr, d = 1.346 g/mL (8); or solid KBr (9). The L_p was withdrawn from the meniscus, overlaid with a salt solution of equilibrium density, recentrifuged as before, transferred to a 5-mL volumetric flask and diluted to mark with phosphate buffered 0.15 M NaCl, pH 7.40 (PBS). Purity was checked by electrophoresis (10).

Chemical, rather than enzymatic, methods of lipid analysis were used in order to avoid the need to dialyze the fractions which would result in substantial loss of material. It was determined that the salt concentration resulting when any L_p fraction was diluted to 5 mL with PBS did not interfere with electrophoresis, with the lipid extractions, nor with chemical analysis of the lipids and the proteins.

The cholesterol contents of the L_p fractions and of whole serum were determined chemically according to Searcy and Bergquist (11). Triglyceride concentrations were determined by the method of Foster and Dunn (12). For PL and cholesteryl ester (CE) analyses, lipids were extracted from serum and from L_p fractions as described by Kates (13). PL were determined as above (6). CE were separated by TLC using the adsorbent described above, but the solvent system was hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol). In addition to separating HDL subfractions by ultracentrifugation, a 1.0-mL aliquot of serum was used to separate HDL by precipitation with the heparin-$MnCl_2$ reagent described by Warnick and Albers (14). Protein in the L_p fractions was determined by the method of Lowry *et al.* (15). Bovine serum albumin was used as standard. Apoproteins A-I (apoA-I) and B (apoB) were determined in whole serum (16). ApoB was also determined in LDL, and apoA-I was determined in HDL_3.

Since enzymatic procedures are most frequently used for cholesterol analysis in aqueous media, we compared values of TSC and of HDLC which were obtained by the chemical method (11) and by an enzymatic procedure (Procedure No. 352, Sigma Diagnostics, St. Louis, MO). Fourteen human serum samples were used in which TSC ranged from 3.62 to 8.60 mmol/L; and HDLC, from 0.78 to 1.81 mmol/L. The chemically determined TSC was lower than the enzymatic by 0.13 ± 0.26 mmol/L. Chemically determined HDLC was lower by 0.11 ± 0.09 mmol/L.

Differences among treatment groups were assessed by one-way analysis of variance. Group means were considered to be significantly different at $P \leq 0.05$ as determined by the least significant difference technique (17).

RESULTS

In our experimental design, the test lipids were ingested as supplements to the habitual diet of the subjects. The subjects were instructed to keep their habitual (basal) diet relatively constant throughout the investigation. In order to monitor this instruction, each man kept a three-day diet record during each test period. These records were used to estimate the energy and nutrient compositions of the basal diets. The average daily energy intake for these subjects was 9123 ± 515 kJ (range: 73500–11,400 kJ). Their average fat consumption was 36 ± 2 en%, not including the test lipids. The test lipids contributed about 450 kJ to the daily energy consumption, approximately 5% of the daily average. Carbohydrate and protein contributed approximately 46 en% and 17 en%, respectively. Cholesterol intake for these subjects was about 300 mg/d. We found no significant interactions of habitual intakes of total energy and fat (not including lipid supplements), diet period and serum lipid concentrations.

The test lipids ingested by the subjects in this study were enclosed in opaque gelatin capsules. Using capsules as vehicles for these test materials had several advantages: (i) the subjects could not identify the particular preparation that they were taking, (ii) these free-living, active men were able to follow the treatment plan at home or while traveling, and (iii) the quantity of each test lipid taken by each man was defined by the number of capsules he took each day and not by measuring each daily dose individually.

The parameters measured in whole serum are shown in Table 2. These included the concentrations of total cholesterol, HDL cholesterol, triglycerides, phospholipids, apoB and apo A-I in blood obtained at baseline and at the

TABLE 2

Lipid and Lipoprotein Contents of Serum of Men Ingesting Dietary Egg Phospholipids, Soybean Phospholipids or Oils with Similar Fatty Acid Compositions[a]

Parameter	Baseline	Treatments			
		EP	EPTG	SP	SPTG
Total serum					
Cholesterol (mmol/L)	4.99 ± 0.52[b,d]	5.79 ± 0.67[c]	5.34 ± 0.75[b,c]	4.78 ± 0.44[d]	4.96 ± 0.52[b,d]
Phospholipids (mmol/L)	2.44 ± 0.20[b]	2.56 ± 0.44[b]	2.40 ± 0.36[b,c]	2.13 ± 0.32[c]	2.53 ± 0.29[b]
Triglycerides (mmol/L)	1.28 ± 0.60	1.18 ± 0.63	1.47 ± 0.79	1.36 ± 0.67	1.42 ± 1.04
HDL cholesterol[e] (mmol/L)	0.96 ± 0.18	1.06 ± 0.18	1.01 ± 0.21	0.98 ± 0.16	1.03 ± 0.18
ApoB (g/L)	0.76 ± 0.12	0.86 ± 0.14	0.79 ± 0.12	0.78 ± 0.11	0.84 ± 0.13
ApoA-I (g/L)	1.09 ± 0.18	1.07 ± 0.17	1.11 ± 0.24	1.03 ± 0.15	1.13 ± 0.18
Lipoproteins					
VLDL					
Protein (g/L serum)	0.22 ± 0.13	0.17 ± 0.11	0.21 + 0.13	0.19 ± 0.12	0.17 ± 0.10
CH (mmol/g protein)	1.50 ± 0.34[b]	2.07 ± 0.59[c]	2.04 ± 0.49[c]	2.07 ± 0.47[c]	1.83 ± 0.54[c]
PL (mmol/g protein)	2.44 ± 0.55	2.85 ± 0.67	2.52 ± 0.61	2.37 ± .011	2.28 ± 0.44
TG (mmol/g protein)	3.19 ± 0.93	3.23 ± 0.75	3.51 ± 0.90	3.92 ± 0.94	3.77 ± 0.63
LDL					
Protein (g/L serum)	0.70 ± 0.12[b]	0.93 ± 0.16[c]	0.77 ± 0.22[b,c]	0.75 ± 0.16[b]	0.82 ± 0.18[b,c]
CH (mmol/g protein)	3.07 ± 0.36	3.41 ± 0.36	3.28 ± 0.36	3.28 ± 0.41	3.28 ± 0.34
CE (mmol/g protein)	2.25 ± 0.39	1.81 ± 1.03	2.07 ± 0.96	1.81 ± 0.75	2.35 ± 0.57
PL (mmol/g protein)	1.04 ± 0.12[b]	0.97 ± 0.13[b,c]	1.00 ± 0.13[b,c]	0.95 ± 0.08[c]	1.03 ± 0.13[b,c]
TG (mmol/g protein)	0.62 ± 0.24	0.43 ± 0.11	0.54 ± 0.18	0.50 ± 0.21	0.51 ± 0.16
ApoB (mmol/g protein)	0.57 ± 0.19	0.58 ± 0.22	0.66 ± 0.20	0.55 ± 0.22	0.58 ± 0.16
HDL_2					
Protein (g/L serum)	0.26 ± 0.09[b]	0.21 ± 0.15[b,c]	0.18 ± 0.06[c]	0.15 ± 0.08[c]	0.20 ± 0.06[b,c]
CH (mmol/g protein)	1.29 ± 0.31[b]	1.01 ± 0.44[b,c]	1.01 ± 0.36[b,c]	1.19 ± 0.34[b]	0.83 ± 0.18[c]
CE (mmol/g protein)	1.09 ± 0.36	1.14 ± 0.47	1.16 ± 0.57	1.37 ± 0.54	0.90 ± 0.28
PL (mmol/g protein)	0.60 ± 0.11	0.55 ± 0.16	0.68 ± 0.16	0.36 ± 0.08	0.35 ± 0.09
TG (mmol/g protein)	0.54 ± 0.21	0.53 ± 0.26	0.56 ± 0.28	0.52 ± 0.26	0.55 ± 0.20
HDL_3					
Protein (g/L serum)	1.17 ± 0.18[b]	0.94 ± 0.28[c]	0.78 ± 0.25[c]	0.82 ± 0.18[c]	0.89 ± 0.29[c]
CH (mmol/g protein)	0.39 ± 0.05	0.41 ± 0.08	0.44 ± 0.10	0.36 ± 0.18	0.41 ± 0.08
CE (mmol/g protein)	0.26 ± 0.052	0.41 ± 0.10	0.41 ± 0.10	0.47 ± 0.10	0.47 ± 0.052
PL (mmol/g protein)	0.27 ± 0.04	0.31 ± 0.08	0.37 ± 0.16	0.36 ± 0.08	0.35 ± 0.09
TG (mmol/g protein)	0.14 ± 0.02	0.16 ± 0.05	0.14 ± 0.06	0.17 ± 0.06	0.19 ± 0.09
ApoA-I (g/g protein)	0.32 ± 0.05	0.39 ± 0.05	0.42 ± 0.08	0.43 ± 0.06	0.41 ± 0.05

[a]Mean ± SD. Abbreviations: baseline, before treatment; EP, egg phospholipids; EPTG, triacylglycerol mixture with a fatty acid composition similar to EP; SP, soybean phospholipids; SPTG, triacylglycerol mixture with a fatty acid composition similar to SP; CH, cholesterol; CE, cholesteryl ester; PL, phospholipids; TG, triacylglycerol; apoB, apoprotein B; apoA-I, apoprotein A-I; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

[b,c,d]Values not sharing a common superscript letter are significantly different, $P \leq 0.05$.

[e]Values were the result of determining the cholesterol content of the supernatant following precipitation of very low density lipoproteins and low density lipoproteins (14).

end of each treatment period. Total serum cholesterol was significantly lower after the SP treatment period compared to either the EP or the EPTG treatments. Total serum phospholipid concentration was significantly decreased after the SP treatment compared to treatment with EP or SPTG, but not EPTG.

The compositions of the lipoprotein fractions separated by ultracentrifugation are also presented in Table 2. Since the lipoproteins were not isolated quantitatively, the compositions are related to the protein mass of the isolated fraction rather than to serum volume. Recovery of the lipoproteins was estimated to be 71% which compares reasonably well with Meijer *et al.* (18). In an experiment with rabbits, these workers reported recoveries of 90 ± 15% (mean ± SD, n = 48) for total cholesterol in lipoprotein fractions. They used a similar isolation procedure, but they did not recentrifuge each L_p fraction through solvent of equilibrium density.

The VLDL at baseline contained significantly less cholesterol (CH) than at any of the treatment periods excepting the SPTG. Of course, triglycerides (TG) was the most abundant lipid in the very low density lipoprotein (VLDL), and the level of VLDL TG was not altered by the treatments used in this investigation. However, EP tended to have the lowest TG and the highest PL concentrations. The CH of VLDL combined free and esterified cholesterol.

LDL protein was significantly higher following EP treatment, compared to baseline and to SP. CH was the most abundant lipid in LDL. After the EP and SP treatment periods, the proportion of the total LDL CH that was esterified was substantially less (approx. 20%) than at baseline or after the other treatments. LDL PL were proportionally less abundant at the end of the SP treatment. The TG content of EP LDL tended to be less than for the other treatment groups or for baseline.

The mass of HDL_2 that was isolated was very small. Although HDL_2 is about 40% protein by mass, the protein concentrations of our HDL_2 preparations were in the range 15–26 mg/dL. Virtually all of the cholesterol was esterified, and the cholesterol content of SP HDL_2 was

significantly greater than for the SPTG HDL_2. This increase in HDL_2 CH was in conjunction with the significant decrease in TSC following the SP treatment. The PL content of HDL_2 was significantly higher for the SP treatment compared to all other treatment conditions. TG content of HDL_2 was not different among treatment groups.

The compositions of the HDL_3 subfractions showed little response to any of these dietary lipids. The only significant change was the increased level of PL in response to the SP and SPTG treatments compared to baseline.

The fatty acid compositions of the serum phospholipids for the various treatment groups and for baseline were similar. PL in baseline serum contained slightly more 18:1n-9 and slightly less 18:2n-6, 20:4n-6, and 22:6n-3. PL in serum after EP treatment contained significantly more 20:5n-3 than after SP treatment.

DISCUSSION

Relative to the effect of SP on the cholesterol concentration of whole serum, our data were consistent with the findings of Knuiman *et al.* (1). When these latter investigators examined the results of studies designed to test the effect of ingested soybean phospholipids on human cholesterolemia, they concluded that any hypocholesterolemic response to phospholipids could be attributed to its content of linoleic acid, 18:2n-6. In contrast with the responses of hypercholesterolemic guinea pigs (5), in this study we found that treatment with the relatively saturated dietary phospholipids EP provided no indication of improved blood lipid profile. TSC reflected the polyunsaturation of the lipid supplement. For this group of subjects, SP resulted in significantly lower average TSC than did EP. Treatment with SP did not significantly lower TSC, compared to SPTG, although the average tended to be lower. The only other significant effect of SP on total serum lipids was *ca.* 19% decrease in the concentration of PL compared to the effects of either SPTG or EP. The PL of hypercholesterolemic guinea pigs had the same qualitative response to SP compared to EP (5). Consistently, Beynen and Terpstra (19) showed a strong positive correlation between serum cholesterol and PL in humans, rabbits and calves.

Although the usefulness of SP specifically as a hypocholesterolemic agent is doubtful, SP may be antiatherogenic. In our experiment using guinea pigs (5), we found that the cholesterol content of aorta decreased in animals fed the SP-containing diet. The intravenous administration of PC has resulted in the regression of atherosclerosis in rabbits (20), in baboons (21) and in quail (22). In each case the regression of the disease state occurred despite continuation of the atherogenic diet. Free cholesterol is transferred from tissue to phosphatidylcholine-containing liposomes. *In vitro* experiments have shown the process to be more efficient in the presence of apoproteins, especially A-I and E. Even tissue cholesteryl esters appear to be mobilized by these apoproteins (23).

An antiatherogenic effect of dietary phospholipid could result from the relationship between PL and HDL. HDL is inversely related to risk of CHD (2). Recently, a prospective study using a large cohort of participants in the Physicians' Health Study demonstrated the power of HDLC and HDL_2 CH as predictors of myocardial infarction (24). Phospholipids from chylomicrons (CM) (25,26), along with apoproteins and unesterified cholesterol (UC), are precursors of HDL (27). Although dietary phospholipids are hydrolyzed to lysophosphatidylcholine and fatty acids for absorption, reesterification recaptures much of the original structures before PL are incorporated into CM (28).

In 1975 Miller and Miller (29) reported an increase in the body cholesterol pool with decreasing concentrations of plasma HDLC. The body cholesterol pool is strongly and positively associated with CHD. The mechanism which accounts for the antiatherogenicity of HDL is not clearly understood. However, the most widely accepted theory is "reverse cholesterol transport" which was proposed in 1968 by Glomset (30). Accordingly, HDL_3 accepts UC from peripheral tissue as the initial step in the "reverse cholesterol transport" theory (31). This step may involve HDL receptors (32) but more likely it is diffusion-controlled (31), and not mediated by HDL_3 cell-surface binding sites (33). HDL_3 is irreversibly converted to HDL_2 when UC is esterified to CE by lecithin:cholesterol acyltransferase (LCAT), a component of HDL_3 which is activated by apoA-I. LCAT uses PC as the acyl source in producing the larger, less dense HDL_2 particles (27). The transfer of HDL_2 CE to the liver for catabolism probably utilizes several pathways, but VLDL appears to be the preferential acceptor of CE. VLDL apoproteins B_{100} and E are able to interact with cell surface receptors for the transfer of CE into the cell (31).

Another possible relationship between dietary phospholipids and HDL_2 could involve cholesterol absorption. When Beil and Grundy (28) investigated the effect of infused PC on human plasma lipoproteins, they were surprised to find that PC blocked cholesterol absorption in the upper part of the small intestine. Other investigators have noted a relationship between cholesterol absorption and HDL metabolism. Shepherd *et al.* (34) reported an increase in the plasma HDL_2/HDL_3 ratio and an increase in apoA-I concentration in men treated with cholestyramine for four weeks. Childs *et al.* (35) observed consistent inverse relationships between consumption of certain shellfish (oysters and clams) which resulted in reduced cholesterol absorption and HDL_2/HDL_3 ratio.

Our data suggest a relationship between dietary polyunsaturated phospholipids (SP) and HDL metabolism. Although treatment with SP and SPTG did not result in significantly different TSC, the cholesterol determined in the HDL_2 fraction was significantly higher following SP treatment compared to treatment with SPTG. When we compared cholesteryl ester concentrations in HDL_2 isolated for all treatment groups, the CE for the SP group tended to be higher than for the other groups. Because of large standard deviations, however, these differences were not significant. A difficulty in determining these lipid concentrations, CH and CE, was that the CH concentration combined UC and CE and was determined in the aqueous medium of the isolated fraction. CE and PL were determined in the lipid extract of an aliquot of serum or L_p fraction. The determination of CE was preceded by its separation from the UC by TLC. These additional steps could easily account for the large standard deviations. Since by far the majority of cholesterol in the HDL_2 is esterified, the small differences between the CH and the

CE concentrations were buried in these deviations, Table 2.

Our data revealed no effect on HDL_2 of EP compared to EPTG. Apparently, the fatty acid composition of the dietary phospholipids was important for the HDL_2 response. EP and EPTG are relatively saturated compared to SP and SPTG with P/S ratios of 0.38 and 3.5, respectively. Dietary saturated triglycerides appear to suppress hepatic LDL receptor activity compared to dietary polyunsaturated fat by decreasing the number of hepatic LDL receptors (36,37). However, that would not explain the absence of an HDL_2 response to the EP treatment. Polyunsaturated PC may favor cholesterol esterification.

The possibility that dietary sources of phospholipids could induce increases in HDL_2 is provided by our experiments with men ingesting soybean and egg phospholipids. Further studies with an appropriate animal model should investigate the regulation of HDL metabolism by dietary phospholipids.

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Molecular Species Composition of Glycerophospholipids from White Matter of Human Brain

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The molecular species composition of the major glycerophospholipids from white matter of human brain were determined by high-performance liquid chromatography of the 3,5-dinitrobenzoyl derivatives of the corresponding diradylglycerols. In phosphatidylcholine (PC) and phosphatidylserine (PS), molecular species containing only saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) comprised 85.7 and 82.4% of the respective totals, with 18:0/18:1 predominant in PS and 16:0/18:1 in PC. These molecular species were also abundant in phosphatidylethanolamine (PE), but in this phospholipid species containing polyunsaturated fatty acids (PUFA), largely 18:0/22:6n-3 and 18:0/20:4n-6, accounted for over half the total; 18:1/18:1 was also abundant in PE. In contrast, 1-*O*-alk-1′-enyl-2-acyl *sn*-glycero-3-phosphoethanolamine (GPE) had much more SFA- and MUFA-containing species, predominantly 16:0a/18:1, 18:0a/18:1 and 18:1a/18:1, with low amounts of species containing 20:4n-6 and 22:6n-3. In alkenylacyl GPE, 22:4n-6 was the major PUFA and 16:0a/22:4n-6 and 18:1a/22:4n-6 the main PUFA-containing species. There was six times more 22:6n-3, twice as much 20:4n-6 and half the amount of 22:4n-6 in PE as compared to alkenylacyl GPE.
Lipids 28, 13–17 (1993).

Nervous tissue contains large amounts of phospholipid with a distinctive fatty acid composition. In mammalian brain, 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (plasmalogen) is especially abundant (1), and the glycerophospholipids overall have a fatty acid composition with characteristically high contents of arachidonic, 20:4n-6, docosatetraenoic, 22:4n-6 and docosahexaenoic, 22:6n-3, acids (2). Arachidonate and docosahexaenoate are selectively incorporated into brain tissue during development, but in humans after the age of two years changes in polyunsaturated fatty acids (PUFA) levels are slight (3,4). The rapid changes in brain fatty acid composition in early development are associated with the formation of dendrites and axons by neurons and myelination by oligodendrocytes (5). Neural tissue from adult mammals shows considerable resistance to dietary regimes deficient in PUFA, and depletion of 22:6n-3 in brain can often only be achieved after two generations (6–9). Dietary studies in rhesus monkeys (6) and rats (9) showed that depletion of 22:6n-3 in retina and brain impaired the visual and cognitive abilities of the offspring. Replacement of 22:6n-3 with n-6 PUFA may produce alterations in the biophysical properties of photoreceptor and neuronal membranes which may cause these functional abnormalities.

The fatty acid composition of individual brain glycerophospholipids from many species, including humans (2,3), has been studied; however, molecular species have only been determined in a few cases (10–12). The only information available on phospholipid molecular species of human brain is the presence of phosphatidylcholine (PC) species containing very long-chain fatty acids (13). The fatty acid composition of brain tissue differs markedly depending on the area in the brain sampled, which reflects the type of cell present. White matter largely consists of myelin membranes and oligodendrocyte cell bodies while gray matter contains astrocytes (3). The phospholipids from gray matter contain much more PUFA, especially in phosphatidylethanolamine (PE) and phosphatidylserine (PS), than those from white matter which show a fatty acid composition typical of myelin (2,3). The aim of the present study was to define the molecular species composition of the major glycerophospholipids in white matter from adult human brain.

MATERIALS AND METHODS

Materials. Phospholipase C from *Bacillus cereus* was purchased from Boehringer Corporation (London, U.K.). Butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (Poole, Dorset, U.K.). 3,5-Dinitrobenzoyl chloride was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and was recrystallized from carbon tetrachloride before use. Standard lipids were obtained from Sigma and from Nu-Chek Prep (Elysian, MN), as detailed earlier (14). Merck thin-layer chromatography (TLC) and high-performance TLC (HPTLC) plates coated with silica gel 60, Analar glacial acetic acid, carbon tetrachloride, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, U.K.). All other solvents were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland). Ultrasphere ODS and Ultrasphere C8 HPLC columns (25 ×0.46 cm, 5 μm particle size) were obtained from Altex/Beckman (Beckman Instruments U.K., High Wycombe, Bucks, U.K.).

Tissue samples. Samples of normal brain white matter were obtained from The Multiple Sclerosis Society Brain Bank, Institute of Neurology, London, U.K. White matter from the parietal ventricle was taken from three male patients, aged 53, 54 and 66 years, who had died from cardiac failure. All samples were collected and frozen at −70°C within 10–44 h after the death of the patient. The fatty acid composition of the phospholipid classes from these samples were published as part of a study comparing the lipid composition of white matter from multiple sclerosis and healthy brain (15).

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Abbreviations: BHT, butylated hydroxytoluene; GLC, gas-liquid chromatography; GPE, *sn*-glycero-3-phosphoethanolamine; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TLC, thin-layer chromatography; UV, ultraviolet. Molecular species are abbreviated as follows: *e.g.*, 16:0/18:1 PE is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; the corresponding alkenylacyl species, 1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine is 16:0a/18:1.

Extraction and purification of lipids. All solvents apart from those used for TLC and HPLC (high-performance liquid chromatography) contained 0.01% (wt/vol) BHT. Samples were stored at −20°C under nitrogen between preparative procedures.

Total lipid was extracted from 200–250 mg of each brain sample according to the method of Folch *et al.* (16). Phospholipid classes were separated by TLC using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aq.KCl (25:25:25:10:9, vol/vol/vol) as developing solvent (17). The lipids were visualized under ultraviolet (UV) light after spraying with a solution of 0.1% (wt/vol) 2′,7′-dichlorofluorescein in methanol. Phospholipid bands were scraped from the plate and eluted with chloroform/methanol/water (5:5:1, vol/vol/vol). Solvent was removed by rotary evaporation under vacuum at 35°C. Fatty acid methyl esters (FAME) of the phospholipids were prepared and analyzed using a Canberra Packard 436 gas chromatograph (Caversham, U.K.) equipped with either a CP Wax 52CB or CP Sil 5CB fused silica capillary column (both 50 m × 0.34 mm i.d., Chrompack U.K. Ltd., London, U.K.) as described previously (15).

The fatty acid compositions of individual phospholipid classes from each tissue sample were very similar. The individual classes from the three samples were therefore pooled prior to preparing derivatives for molecular species analysis.

Preparation of 3,5-dinitrobenzoyl derivatives. Two-mg portions of PC and PE and 1-mg portions of PS were hydrolyzed with phospholipase C from *B. cereus* using a two-phase system of 1 mL diethyl ether and 1 mL 0.01M Tris-SO_4, pH 7.4, at room temperature for 5 h (18). At the end of the incubations, 1,2-diradylglycerols were extracted and the diacyl and 1-alkenyl-2-acyl glycerols separated by TLC using hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol) (19). Modern precoated TLC plates separate these sub-classes without resorting to borate impregnated silica. Diradylglycerols were then derivatized in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C for 45 min under nitrogen, extracted and washed as described by Takamura *et al.* (20). The purity of the product was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol).

Separation of molecular species. The 1,2-diradyl-3-dinitrobenzoyl-*sn*-glycerols were separated by HPLC at 19–21°C on reverse phase columns using a Pye Unicam 4010 pump (Pye Unicam, Cambridge, U.K.) and three isocratic solvent systems. An ODS column was used with methanol/propan-2-ol (95:5, vol/vol) at a flow rate of 1.0 mL/min, and acetonitrile/propan-2-ol (80:20, vol/vol) at a flow rate of 1.0 mL/min (20); a C8 column was used with methanol/water/acetonitrile (93:5:2, vol/vol/vol) at a flow rate of 1.2 mL/min (19). Peaks were detected at 254 nm with a Pye Unicam 4020 detector and quantified using a Shimadzu C-R3A recording integrator (Anachem, Luton, Bedfordshire, U.K.).

Peaks were identified from plots of $\log_{10}$ (relative retention time) *vs.* the effective carbon number at the C-1 position of glycerol (20,21) by adding a 16:0/16:0 standard or by using 16:0/22:6 as a reference peak. Peaks on chromatograms were also identified by reference to the fatty acid composition of the samples and to molecular species identified in previous work (14,19). The major alkenyl acyl species were identified from a sample of bovine brain PE by collecting peaks and identifying component FAME and dimethylacetals by gas-liquid chromatography (GLC) following transesterification in 1% (vol/vol) sulfuric acid in methanol. Molecular species containing 20:2n-9, 20:3n-9, 22:3n-9 and 24:4n-6 gave relative retention times in each of the three solvent systems that were consistent with the identifications shown, but insufficient material was available to confirm these identifications directly by GLC analysis of individual molecular species. Each sample was chromatographed three times in each solvent system and the standard deviation calculated. Where final peak areas were calculated by subtraction, the standard deviations of the contributing peaks were added to give the final error. Results are presented to one decimal place for clarity.

RESULTS

The three main diacyl glycerophospholipid classes in human brain showed very different molecular species profiles (Table 1). In PC, saturated fatty acid/monounsaturated fatty acid (SFA/MUFA) species comprised two-thirds of the total with 16:0/18:1 (45.5%) and 18:0/18:1 (21.0%) being predominant. SFA/SFA and MUFA/MUFA species were also abundant with each category, making up almost 10% of PC; 16:0/16:0 and 18:1/18:1 were the main species in each category, respectively. Species containing PUFA were of minor importance. Arachidonic acid was the most abundant PUFA with smaller amounts of 18:2n-6, 22:6n-3, 22:4n-6 and 20:3n-6; 18:0/20:4n-6 was the only PUFA-containing species present at more than 1%.

In contrast, PUFA-containing species were much more abundant in diacyl PE, and the SFA/PUFA class was the largest component with over 40% of the total, mainly 18:0/22:6n-3 (19.3%) and 18:0/20:4n-6 (10.4%). The next most abundant species was 18:0/22:4n-6 (4.4%) with 16:0/22:6n-3, 16:0/20:4n-6, 16:0/22:4n-6 and 18:0/20:3n-9 all present at over 1%. MUFA/PUFA species totaled 9.2%, with 18:1/20:4n-6 contributing over half of this total. MUFA/MUFA species comprised almost a quarter of the total, with 18:1/18:1 contributing most of it (18.8%). SFA/MUFA species comprised almost 20%, but in contrast to PC, 18:0/18:1 (12.3%) was more abundant than 16:0/18:1 (5.5%). No SFA/SFA species were detected. PS was dominated by 18:0/18:1 which comprised 61.2% of the total molecular species. Another SFA/MUFA species, 18:0/20:1, was the next most abundant (9.2%), with 18:1/18:1 being almost equally abundant (8.5%). Only four other species contributed more than 1%, 18:0/22:6n-3, 18:0/20:4n-6, 18:0/22:4n-6 and the unresolved pair 18:1/20:1 + 20:1/18:1.

In plasmalogen PE, the 16:0, 18:1 and 18:0 alkenyl species were all of major importance, and 17:0 alkenyl species were minor components (Table 2). In decreasing order, 22:4n-6, 20:4n-6 and 22:6n-3 were the major PUFA, this being reflected in 16:0/22:4n-6, 18:1/22:4n-6 and 18:1/20:4n-6 being the main PUFA-containing species. Molecular species containing 18:2n-6, 20:3n-9, 22:3n-9, 20:2n-9 and 24:4n-6 were all found in small amounts. The MUFA/MUFA and SFA/MUFA classes both totaled about 29% with 18:1a/18:1 as the single most important species (22.9%). Also abundant were 16:0a/18:1 and 18:0a/18:1, being the only other species present at greater than 10%. SFA/SFA species totaled 5.7%, most of which was 16:0a/16:0.

TABLE 1

Molecular Species Composition of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and Phosphatidylserine (PS) from White Matter of Human Brain[a]

	Molecular species	Mole % PC	Mole % PE	Mole % PS
SFA/PUFA	16:0/22:6	0.3 ± 0.1	2.9 ± 0.1	trace
	16:0/20:4	0.9 ± 0.1	1.1 ± 0.0	—
	16:0/22:4	trace	2.0 ± 0.2	trace
	16:0/18:2	0.4 ± 0.2	0.5 ± 0.1	—
	18:0/22:6	0.4 ± 0.1	19.3 ± 0.2	3.4 ± 0.3
	18:0/20:4	1.9 ± 0.0	10.4 ± 0.1	1.7 ± 0.1
	18:0/22:4	trace	4.4 ± 0.5	3.1 ± 0.0
	18:0/18:2	0.3 ± 0.0	0.5 ± 0.3	—
	18:0/20:3	0.4 ± 0.0	1.1 ± 0.0	0.6 ± 0.0
	18:0/20:2	—	—	0.4 ± 0.0
	Total	4.6	42.7	9.2
MUFA/PUFA	18:1/22:6	trace	1.5 ± 0.1	—
	18:1/20:4	0.5 ± 0.0	5.0 ± 0.1	0.3 ± 0.1
	18:1/22:4	trace	0.6 ± 0.2	0.4 ± 0.0
	18:1/18:2	0.3 ± 0.0	0.4 ± 0.2	—
	20:1/22:6	—	1.0 ± 0.1	—
	20:1/20:4	0.5 ± 0.1	0.4 ± 0.1	trace
	20:1/22:4	—	0.3 ± 0.1	0.3 ± 0.1
	Total	1.3	9.2	1.0
SFA/SFA	16:0/16:0	7.4 ± 0.2	—	—
	16:0/18:0; 18:0/16:0	1.9 ± 0.2	—	—
	Total	9.3	—	—
SFA/MUFA	16:0/18:1; 18:0/16:1	45.5 ± 0.8	5.5 ± 0.0	0.4 ± 0.0
	18:0/18:1; 16:0/20:1	21.0 ± 0.6	12.3 ± 0.1	61.2 ± 2.8
	18:0/20:1	0.5 ± 0.2	2.0 ± 0.1	9.2 ± 0.2
	Total	67.0	19.8	70.8
MUFA/MUFA	16:1/18:1; 18:1/16:1	0.7 ± 0.1	0.5 ± 0.0	—
	18:1/18:1; 16:1/20:1	6.9 ± 0.1	18.8 ± 0.3	8.5 ± 0.2
	18:1/20:1; 20:1/18:1	1.0 ± 0.1	3.7 ± 0.1	2.3 ± 0.1
	20:1/20:1	0.1 ± 0.0	0.4 ± 0.0	0.7 ± 0.1
	18:1/24:1; 24:1/18:1	0.3 ± 0.1	—	0.1 ± 0.0
	20:1/24:1; 24:1/20:1	0.4 ± 0.1	—	—
	Total	9.4	23.4	11.6
	Unknowns	6.1 (7)	4.7 (6)	7.0 (7)

[a]Values are means ±1 SD of three determinations of a pooled sample from three individuals, rounded to the nearest decimal place. Saturated fatty acid/monounsaturated fatty acid (SFA/MUFA) molecular species may contain small amounts of the reverse isomer, but for convenience the SFA is shown at the C-1 position of the glycerol; —, not detected; trace, <0.1%. The following fatty acid isomers were identified by gas-liquid chromatography: 16:1, n-7 and n-9; 18:1, n-7 and n-9; 20:1, n-7 and n-9; 24:1n-9; 18:2n-6; 20:2n-9; 20:3, n-6 and n-9; 20:4n-6; 22:4n-6; 22:6n-3 (ref. 15). PUFA, polyunsaturated fatty acid.

DISCUSSION

The glycerophospholipids from mature, adult human brain white matter contain more MUFA and less SFA and PUFA than do gray matter (2,3). It is therefore not surprising that the SFA/MUFA and MUFA/MUFA categories were major components in all three diacyl classes of phospholipid from white matter; there was also 9% of SFA/SFA in PC. There was only 5.9% of PUFA-containing species in PC and 10.2% in PS, the latter phospholipid class being dominated by 18:0/18:1 (61.2%). PC from rat cerebellum also contained low amounts of PUFA-containing molecular species and was dominated by 16:0/16:0, 16:0/18:1 and 18:0/18:1, which together totaled 67.5% (11). Earlier studies on human brain of different ages showed about twice as much PUFA in PC from gray matter than in PC from white matter (2,3). The fatty acids of myelin PC from ox brain and mouse brain also contained only 1.0% and 3.3% PUFA, respectively (22), while the fatty acids of PC from the cerebral cortex of rhesus monkeys contained 5.9% n-6 PUFA and 5.1% n-3 PUFA (23). These investigators also found low levels of PUFA in PS (22,23). In contrast, PC from fish brain contains much larger amounts of 22:6n-3, and 16:0/22:6n-3 was a major molecular species of brain PC from trout and cod (14,24). However, 16:0/18:1 and 18:0/18:1 were also abundant, whereas 18:1/24:1 + 24:1/18:1 comprised about 10% of fish brain PC (14,24). To our knowledge the latter molecular species has not been found in other animals. Brain PC, therefore, is much more saturated than PC from other tissues in terrestrial mammals or in fish. These differences in composition between animal species are even more marked in PS where 18:0/22:6n-3, 18:1/22:6n-3 and 22:6n-3/22:6n-3 are all abundant in brains of trout and cod

TABLE 2

Molecular Species of 1-*O*-alk-1′-enyl-2-acyl GPE from White Matter of Human Brain[a]

	Molecular species	Mole %
SFA/PUFA + MUFA/PUFA	16:0a/22:6	0.8 ± 0.1
	18:1a/22:6	0.6 ± 0.0
	16:0a/20:4	1.0 ± 0.0
	18:1a/20:4	4.6 ± 0.1
	16:0a/22:4	7.6 ± 0.4
	18:1a/22:4	6.8 ± 0.2
	16:0a/18:2 + 18:1a/18:2	0.9 ± 0.1
	16:0a/20:3 + 18:1a/20:3	0.9 ± 0.7
	16:0a/22:3 + 18:1a/22:3	2.5 ± 0.1
	16:0a/20:2 + 18:1a/20:2	1.6 ± 0.2
	16:0a/24:4 + 18:1a/24:4	2.2 ± 0.3
	18:0a/22:6	2.3 ± 0.1
	18:0a/20:4	2.4 ± 0.1
	18:0a/22:4	2.1 ± 0.2
	18:0a/20:3	0.4 ± 0.1
	Total	36.7
SFA/SFA	16:0a/16:0	3.9 ± 0.1
	17:0a/16:0	0.5 ± 0.1
	17:0a/18:0	0.4 ± 0.0
	16:0a/18:0 + 18:0a/16:0	0.7 ± 0.1
	18:0a/18:0	0.2 ± 0.1
	Total	5.7
SFA/MUFA	16:0a/18:1 + 18:1a/16:0	15.5 ± 1.5
	17:0a/18:1	1.0 ± 0.1
	17:0a/20:1	0.3 ± 0.0
	18:0a/18:1; 16:0a/20:1	10.6 ± 1.7
	18:0a/20:1; 16:0a/22:1	1.7 ± 0.1
	18:0a/22:1	0.1 ± 0.0
	Total	28.4
MUFA/MUFA	18:1a/18:1	22.9 ± 1.1
	18:1a/20:1	5.5 ± 0.1
	18:1a/22:1	0.7 ± 0.1
	Total	29.1

[a]Values are means ±1 SD of three determinations of a pooled sample from three individuals, rounded to the nearest decimal place. The following fatty acid and dimethyl acetal isomers were found by gas-liquid chromatography: 18:1, n-7 and n-9; 18:1a, n-7 and n-9; 20:1, n-7 and n-9; 22:1n-9; 18:2n-6; 20:2n-9; 20:3, n-6 and n-9; 20:4n-6; 22:3n-9; 22:4n-6; 22:6n-3; 24:4n-6 (ref. 15). It was not possible to separate 16:0 alkenyl and 18:1 alkenyl pairs containing 18:2, 20:2, 20:3, 22:3 and 24:4 because of the presence of 17:0 alkenyl species. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid.

(14,24). Interestingly, Connor *et al.* (23) found PS (and PE) of brain of rhesus monkeys to be much more amenable to dietary perturbations than PC.

In the present study both PE and alkenylacyl *sn*-glycero-3-phosphoethanolamine (GPE) were much more unsaturated than were PC and PS, confirming earlier studies (2,3), but the PUFA compositions of the two subclasses of ethanolamine glycerophospholipid were markedly different. In the human brain PE samples analyzed here, 18:0/22:6n-3 and 18:0/20:4n-6 were major species, but there was a higher proportion of 18:1/18:1 and 18:0/18:1 and lower proportions of PUFA-containing species than in either ox or rat (10,11). In PE from bovine brain 18:0/22:4n-6 was also important and in rat brain 16:0/22:6n-3 comprised over 10% of the total (10,11). In trout and cod brain PE, di22:6n-3, 16:0/22:6n-3, 18:1/22:6n-3 and 18:0/22:6n-3 were the four main species, totaling 58–61% (14,24).

In the brains analyzed here, alkenylacyl GPE was more saturated than PE with PUFA-containing molecular species totaling 36.7% in the former *vs.* 51.9% in the latter; 18:1a/18:1, 16:0a/18:1 and 18:0a/18:1 were all major components of alkenylacyl PE. An early study of ox and mouse brain myelin had shown the preponderance of 22:4n-6 in PE relative to other glycerophospholipids (22), and in a later study the molecular species of PE subclasses in bovine brain showed a concentration of 22:4n-6 containing species in the alkenylacyl fraction (10), *i.e.*, the same as found here in humans. In juvenile rhesus monkeys a diet supplemented with safflower oil caused large increases in 22:4n-6 and 22:5n-6 in total PE at the expense of 22:6n-3 (23). An early study by O'Brien and Sampson (3) found more 22:5n-6 than 22:6n-3 in human brain white matter and myelin, with very small amounts of 22:4n-6. However, subsequent studies in a wide range of animal species found 22:4n-6 to be the major C_{22} n-6 PUFA with

little, if any, 22:5n-6 (2,4,5,6,15,22); it seems probable that 22:5n-6 was misidentified in the original work. It is possible that 22:4n-6 is a poor substrate for the Δ4 desaturase, or that 22:5n-6 is no better than 22:4n-6 or 22:6n-3 in fulfilling the role of these PUFA in brain cells. Alkenylacyl GPE also contained a number of minor PUFA, including 20:2n-9, 20:3n-9, 22:3n-9 and 24:4n-6. Some of these 16:0a/PUFA + 18:1a/PUFA pairs could not be resolved. There is, therefore, a marked concentration of n-6 PUFA in alkenylacyl GPE relative to PE, and the converse is true for 22:6n-3. The significance of this difference is not yet understood.

The presence of n-9 PUFA, albeit in small amounts, is intriguing since these fatty acids are usually taken to indicate essential fatty acid deficiency. Small amounts of 20:3n-9 were also reported in bovine brain (10,22). Neural fatty acid composition responds to changes in dietary fat in experimental animals even after the rapid accumulation of fatty acids during growth has ceased (23,25), and it is possible that there are differences in brain fatty acid composition in humans depending on dietary history. Dietary therapy to alleviate the symptoms of adrenoleukodystrophy is known to cause changes in brain fatty acid compositon (26).

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1-*O*-Alk-1′-enyl-2-acyl-glycerophosphoethanolamine Content and Molecular Species Composition in Fish Brain

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Ethanolamine glycerophospholipids from the brains of both trout and cod comprised 36–38% of 1-*O*-alk-1′-enyl-2-acyl-glycerophosphoethanolamine (GPE) determined using two methods. In 1-*O*-alk-1′-enyl-2-acyl-GPE from trout brain, the main molecular species were 18:1a/18:1, 18:0a/18:1 and 16:0a/18:1, which totalled 63.3%, while polyunsaturated fatty acid (PUFA) containing species totalled only 18.2%. 1-*O*-Alk-1′-enyl-2-acyl-GPE from cod brain was much more unsaturated with PUFA containing species totalling 52.6%, of which 18:0a/20:5n-3, 18:1a/20:5n-3 and 18:1a/22:6n-3 were predominant. In cod 18:1a/18:1, 18:0a/18:1 and 16:0a/18:1 were the only other species present at over 5% each, totalling 31.8%. In both cod and trout, small amounts of species containing 22:4n-6 were found. The results of this and earlier studies indicate that there is considerable specificity of composition at the level of molecular species between different lipid classes and subclasses.
Lipids 28, 19–22 (1993).

Molecular species determinations of glycerophospholipids have revealed the full complexity of the lipid constituents of the biomembrane at the molecular level. However, most analyses have been of the diacyl classes of the major glycerophospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Data for ethanolamine plasmalogens are more limited but have been obtained for bovine brain (1), rat brain (2) and monkey brain (3). Changes in molecular species composition during brain development (2) and the influence of diet on the lipid composition of brain (3) have also been investigated. Complete molecular species analyses of 1-*O*-alk-1′-enyl-2-acyl-glycerophosphoethanolamine (GPE) have been published for platelets (4), red blood cells (5) and umbilical vein and artery cells (6) from humans, neutrophils (7) and red blood cells (8) from rats, and macrophages from mice (9).

The 1-*O*-alk-1′-enyl-2-acyl-GPE fraction of ethanolamine glycerophospholipids (EGP) is a major component of mammalian brain (10). As part of a study investigating the role of polyunsaturated fatty acids (PUFA) in neural tissue, the subclass composition of EGP was determined in the brains of a freshwater fish, rainbow trout (*Salmo gairdneri*) and a marine fish, cod (*Gadus morhua*). The molecular species composition of the 1-*O*-alk-1′-enyl-2-acyl-GPE fractions were then analyzed. Previous studies had determined the molecular species composition of the diacyl classes of glycerophospholipids from the brains of these fish (11–13).

MATERIALS AND METHODS

Materials. Rainbow trout (400–600 g weight), obtained from a commercial fish farm, were maintained in a freshwater aquarium at 4–6°C on a standard diet (Ewos Ltd., Westfield, West Lothian, Scotland). Cod (300–600 g weight) were obtained from the Marine Station, Millport (Firth of Clyde, Scotland), maintained in a seawater aquarium at 10–14°C on a diet of chopped squid and used within two weeks of capture. Fish were killed by decapitation; the brains were removed and used immediately.

Phospholipase C from *Bacillus cereus* was obtained from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, England). Butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (Poole, Dorset, England). 3,5-Dinitrobenzoylchloride was from Aldrich Chemical Co. (Gillingham, Dorset, England) and was recrystallized from carbon tetrachloride before use. Standard lipids were obtained from Sigma and from Nu-Chek Prep (Elysian, MN), as detailed previously (11). Analar grade glacial acetic, carbon tetrachloride, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, England). All other solvents of high-performance liquid chromatography (HPLC) grade were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland). Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) plates coated with silica gel 60 (Merck, Darmstadt, Germany) were also from BDH Ltd.

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

Extraction and purification of lipids. All solvents, apart from those used for TLC and HPLC, contained 0.01% (wt/vol) BHT. Samples were stored at −20°C under nitrogen between preparative procedures.

The brains were removed from 10 trout and 20 cod, the meninges separated and the remaining tissue homogenized in 30 mL of chloroform/methanol (2:1, vol/vol) using a polytron tissue disrupter. Total lipid was extracted essentially by the method of Folch *et al.* (14). Phospholipids were separated by TLC using methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol) (15) and fractions were made visible under ultraviolet (UV) light after spraying with 0.1% (wt/vol) 2′,7′-dichlorofluorescein in methanol containing 0.01% (wt/vol) BHT. Ethanolamine glycerophospholipids were eluted from the silica gel with three 40-mL washes of chloroform/methanol/water (5:5:1, vol/vol/vol), and the extract was rotary evaporated under vacuum at 35°C and dried under a stream of nitrogen. 2′,7′-Dichlorofluorescein was removed from lipid extracts by washing with a solution of 2% (wt/vol) $KHCO_3$.

Fatty acid methyl esters were prepared by esterification in 2 mL of 1% (vol/vol) H_2SO_4 in methanol at 50°C under nitrogen for 16 h and chromatographed on a Packard 436

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Abbreviations: BHT, butylated hydroxytoluene; EGP, ethanolamine glycerophospholipids; GLC, gas-liquid chromatography; GPE, glycero-3-phosphoethanolamine; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; UV, ultraviolet. Molecular species of 1-*O*-alk-1′-enyl-2-acyl-GPE are abbreviated as follows: *e.g.*, 16:0a/18:1 GPE is 1-*O*-hexadec-1′-enyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. The corresponding diacyl species, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, is abbreviated as 16:0/18:1.

GLC fitted with a CP Wax 52CB fused-silica capillary column (50 m × 0.32 mm internal diameter) (Chrompack U.K. Ltd., London, England) using hydrogen as carrier gas (16).

Quantitation of alkenylacyl-GPE: Method 1. 1-*O*-Alk-1′-enyl-2-acyl-GPE was analyzed using a modification of the method by Touchstone *et al.* (17) which involves hydrolyzing the 1-*O*-alk-1′-enyl moiety with dilute acid on the origin of an HPTLC plate, separation of the 2-lyso-GPE and diacyl-GPE by chromatography, followed by detection using a phosphate stain and quantitation by densitometry. Eight μg of EGP was streaked on a 5 mm origin on an HPTLC plate, and before the solvent had completely evaporated 2.5 μL of 2.5% (wt/vol) trichloroacetic acid in 1 M HCl was applied over this, followed by a further 2.5 μL 10 min later. The HPTLC plate was air dried then desiccated for 4 h *in vacuo*. The plate was developed twice to 1 cm with methyl acetate/propan-2-ol/chloroform/methanol/0.25% (wt/vol) aq. KCl (25:25:25:10:9, by vol), air dried and then fully developed in this solvent. The plate was dried, lightly sprayed with a phosphate detection reagent (18) and peaks were quantitated by densitometry at 650 nm (Shimadzu CS9000 flying spot densitometer, V.A. Howe Ltd., Banbury, Oxon, England). This method gave a linear response up to 10 μg/component.

Method 2. The 3,5-dinitrobenzoyl derivatives of the total EGP fraction were separated into diradyl classes by HPTLC using hexane/diethyl ether (7:3, vol/vol) as developing solvent. Peaks were quantitated by densitometry at 254 nm. This method also gave a linear response up to at least 10 μg/component.

Preparation of 3,5-dinitrobenzoyl derivatives. Four-mg portions of total EGP were hydrolyzed with 200 units of phospholipase C using a two-phase system of 1 mL diethyl ether and 1 mL 0.1 M sodium borate buffer, pH 7.5, at room temperature for 3 h under nitrogen (19). At the end of the incubations, the 1,2-diradylglycerols were extracted and the 1-*O*-alk-1′-enyl-2-acyl fraction purified by TLC in hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). Amounts of phospholipid remaining after phospholipase digestion were less than 2% of the starting material. 1-*O*-Alk-1′-enyl-2-acyl-glycerols were derivatized in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C for 45 min under nitrogen, extracted and washed as described by Takamura *et al.* (20). The purity of the product was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol).

Separation of molecular species. The 1-*O*-alk-1′-enyl-2-acyl-3-dinitrobenzoyl-*sn*-glycerols were separated by HPLC at 19–21°C on reverse phase columns using a Pye Unicam (Cambridge, England) 4010 pump and three isocratic solvent systems as described previously (21). An ODS column was used with methanol/propan-2-ol (95:5, vol/vol) at a flow rate of 1.0 mL/min and acetonitrile/propan-2-ol (80:20, vol/vol) at a flow rate of 1.0 mL/min; an octyl column was used with methanol/acetonitrile/water (93:5:2, vol/vol/vol) at a flow rate 1.2 of mL/min. Peaks were detected at 254 nm with a Pye Unicam 4020 detector and quantified uing a Shimadzu C-R6A recording integrator (Anachem, Luton, England).

Peaks were identified from plots of $\log_{10}$ (relative retention time, RRT) *vs.* the effective carbon number on the *sn*-1 position of the glycerol as described by Patton *et al.* (22) using 16:0/22:6 diacyl and 16:0/16:0 diacyl as reference peaks. The effective carbon numbers of the alkenyl moieties on these plots were found by chromatography of the 3,5-dinitrobenzoyl derivatives of bovine brain ethanolamine plasmalogen and by identifying the peaks by gas-liquid chromatography (GLC) of the dimethylacetals and fatty acid methyl esters. Each of the samples 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.

RESULTS AND DISCUSSION

Methods used to quantitate the subclass composition of EGP must depend on detecting a constituent which is common to each subclass such as the phosphate group. Alternatively they rely on modifying or replacing the head group with one which can easily be detected. Charring and densitometry of diradyl glycerols or their derivatives following HPTLC separations cannot be used since the subclasses of EGP have variable unsaturation which has a profound effect on staining and consequently quantitation; diacyl-GPE from fish brain contains large amounts of 22:6n-3 (11,13). The presence of dimethylacetals in GLC analyses of fatty acid methyl esters is only an indication of PE plasmalogen since the production of dimethylacetals is rarely quantitative (23).

The first method used in this study to quantitate the 1-*O*-alk-1′-enyl-2-acyl-GPE fraction from fish brain is a simple and quick procedure in which as little as 5 μg of material can be used if diacyl-GPE and 1-*O*-alk-1′-enyl-2-acyl-GPE are present in roughly similar amounts. However, none of the TLC solvents available for separating polar lipid classes can resolve EGP sub-classes and thus 1-*O*-alkyl-2-acyl-GPE cannot be separated from diacyl-GPE. The phosphate stain leaves a slightly blotchy background on the HPTLC plate which probably accounts for much of the error in quantitating the fractions.

The second method which was used to verify the validity of the approach described above utilizes the UV absorbing properties of the dinitrobenzoyl group to give quantitation independent of acyl unsaturation at the wavelength used. The preparation of dinitrobenzoyl derivatives is relatively time-consuming and also requires more material, but has the advantage that the diacyl and 1-*O*-alkyl-2-acyl subclasses can be resolved.

The two procedures outlined above gave identical results for the content of 1-*O*-alk-1′-enyl-2-acyl-GPE within the experimental error of the methods (Table 1). The values obtained for the two species were similar (Table 1) but substantially lower than the values reported for mammalian brain which typically contains over 50% of PE as plasmalogen (10). However, the different methodologies employed in many earlier studies may mean that such comparisons are not entirely valid. In addition, Method 2 shows 2–3% of 1-*O*-alkyl-2-acyl-GPE was present in the EGP fraction from both species (Table 1), slightly lower than the proportion found in mammalian brain (10).

The alkenyl moieties in 1-*O*-alk-1′-enyl-2-acyl-GPE from the brains of trout and cod were predominantly 16:0a, 18:0a and 18:1a with very small amounts of 17:0a present in trout and slightly more in cod (Table 2). The molecular species compositions showed some large differences be-

TABLE 1

Diradyl Class Composition of Ethanolamine Glycerophospholipids from the Brain of Trout and Cod

	Trout (mole %)		Cod (mole %)	
	Method 1[a]	Method 2[b]	Method 1[a]	Method 2[b]
1,2-Diacyl-GPE	63.9 ± 5.5	59.3 ± 0.9	61.4 ± 3.5	60.0 ± 1.2
1-*O*-Alk-1′-enyl-2-acyl-GPE	36.1 ± 5.5	38.5 ± 1.1	38.6 ± 3.5	37.2 ± 1.1
1-*O*-Alkyl-2-acyl-GPE	—[c]	2.1 ± 0.7	—[c]	2.8 ± 0.3

[a]1-*O*-Alk-1′-enyl-2-acyl-GPE was hydrolyzed with dilute acid on the origin of an high-performance thin-layer chromatography (HPTLC) plate, 2-lyso-glycero-3-phosphoethenolamine (GPE) was separated from phosphatidylethanolamine + 1-*O*-alkyl-2-acyl-GPE, the spots visualized with a phosphate stain and quantitated by densitometry as detailed in Materials and Methods.

[b]3,5-Dinitrobenzoyl derivatives of the total ethanolamine glycerophospholipids fraction were separated into classes by HPTLC and quantitated by densitometry as detailed in Materials and Methods.

[c]1,2-Diacyl-GPE includes 1-*O*-alkyl-2-acyl-GPE since Method 1 cannot separate these subclasses.

tween the two species of fish (Table 2) with that from cod brain being much more unsaturated. The saturated alkenyl/PUFA and monounsaturated alkenyl/PUFA subclasses were 18.2% and 16.2% more abundant, respectively, in cod than in trout with 18:0a/20:5n-3, 18:1a/20:5n-3 and 18:1a/22:6n-3 all present at over 5% in cod (Table 2). In contrast, molecular species containing only saturated or monounsaturated moieties comprised 75.8% in trout compared with 43.8% in cod (Table 2); 18:1a/18:1, 16:0a/18:1 and 18:0a/18:1 were the most abundant molecular species in both trout and cod.

In tissues from terrestrial animals the 1-*O*-alk-1′-enyl-2-acyl subclass of EGP is usually more polyunsaturated than the diacyl subclass *e.g.*, in mouse macrophage (9), rat neutrophil (7), rat erythrocyte (8), rat cerebellum (2), ox and mouse brain myelin (24), contrary to the case in fish brain, especially with respect to trout. The composition of 1-*O*-alk-1′-enyl-2-acyl-GPE is very different from that of PE in both trout and cod brain, where PE was very unsaturated, containing about 14% of di22:6n-3, and only 16–20% of molecular species comprised only saturated and monounsaturated moieties (11,13). In PE from fish brain, 22:6n-3 is by far the major PUFA, yet in 1-*O*-alk-1′-enyl-2-acyl-GPE from trout brain 22:6n-3, 20:4n-6, 22:5n-3 and 22:4n-6 are all of similar low abundance. In 1-*O*-alk-1′-enyl-2-acyl-GPE from cod brain, 20:5n-3 is the major PUFA with 18:0a/20:5n-3 (10.8%) and 18:1a/20:5n-3 (7.9%) being the two most important PUFA containing species (Table 2). Therefore, cod brain 1-*O*-alk-1′-enyl-2-acyl-GPE is similar to phosphatidylinositol (PI) from both trout and cod brains in that it is dominated by 18:0/20:5n-3 species (11,12). This selectivity may indicate a special role for 20:5n-3 in fish brain.

The presence of small amounts of molecular species containing 22:4n-6 in 1-*O*-alk-1′-enyl-2-acyl-GPE from both trout and cod brain is unexpected since this fatty acid was not found in any diacyl glycerophospholipid class in brain from either species (11–13). However, several studies in other organisms have found 22:4n-6 to be more abundant in the 1-*O*-alk-1′-enyl-2-acyl subclass of EGP than in diacyl-GPE, *e.g.*, in rat testes (25), rat brain (2,26), synaptosomal and myelin fractions from mouse brain (24), myelin from ox brain (27), and brain from monkeys (3) and humans (28). Sometimes the difference is an order of magnitude, *e.g.*, in rat erythrocyte (8) and mouse macrophage (9). This marked selectivity suggests that 22:4n-6 when paired with a 1-*O*-alk-1′-enyl group must be particularly suited for a specific role in membrane structure.

1-*O*-Alk-1′-enyl-2-acyl-GPE from both trout and cod brains has about three times more n-6 PUFA and 2.5 to 7 times less n-3 PUFA than the corresponding diacyl-GPE fraction (11,13). Consequently, the n-3/n-6 ratios of the diacyl- and 1-*O*-alk-1′-enyl-2-acyl-GPE subclasses are very different; 52.8 *vs.* 2.6 and 26.9 *vs.* 4.4 in trout and cod, respectively. It is well known that the high n-3/n-6 ratio found in fish lipids is a reflection of a high dietary input of n-3 PUFA, but it is clear from this study that 1-*O*-alk-1′-enyl-2-acyl-GPE is markedly depleted of n-3 PUFA and enriched in n-6 PUFA relative to diacyl-GPE; the selectivity for 22:4n-6 in 1-*O*-alk-1′-enyl-2-acyl-GPE is particularly impressive.

In a study of glycerophospholipid molecular speciation in human brain, which contains much more n-6 PUFA and less n-3 PUFA, the n-3/n-6 ratios of diacyl-GPE and 1-*O*-alk-1′-enyl-2-acyl-GPE were 0.96 and 0.13, respectively (28). However in brain from monkeys fed a control (soy oil) diet, the PUFA compositions of both EGP subclasses were similar and the n-3/n-6 ratios close to unity (3). In monkeys the PUFA composition of both EGP subclasses was easily perturbed by dietary manipulation; thus when monkeys were fed diets containing safflower oil or fish oil, the n-3/n-6 ratios of both subclasses changed by similar amounts to 0.12 and 2.4–3.1, respectively (3).

The compositional plasticity in response to dietary input can also be observed in blood cells from humans. The major molecular species of 1-*O*-alk-1′-enyl-2-acyl-GPE in human platelets are 18:0a/20:4n-6, 16:0a/20:4n-6 and 18:1a/20:4n-6 (4), but when subjects were fed a diet enriched in eicosapentaenoic acid, the 1-*O*-alk-1′-enyl-2-acyl subclass of EGP was found to be most enriched in this fatty acid and accounted for the largest reservoir of EPA in platelets (29). Some studies have also shown 1-*O*-alk-1′-enyl-2-acyl-GPE to be metabolically more active than other glycerophospholipids. In human platelets stimulated with thrombin and collagen, arachidonic acid-con-

TABLE 2

Molecular Species Composition of 1-*O*-Alk-1′-enyl-2-acyl-GPE from the Brain of Trout and Cod[a]

	Trout (mole %)	Cod (mole %)
16:0a/20:5	0.5 ± 0.0	2.4 ± 0.1
16:0a/22:6	1.7 ± 0.5	1.7 ± 0.1
16:0a/20:4	1.2 ± 0.2	0.8 ± 0.1
16:0a/22:5	—	0.9 ± 0.2
16:0a/22:5 + 18:1a/20:4	2.9 ± 0.3	—
16:0a/22:4 + 18:1a/22:4	1.0 ± 0.4	3.0 ± 0.4
17:0a/22:6	—[b]	2.0 ± 0.4
18:0a/20:5	2.3 ± 0.5	10.8 ± 0.9
18:0a/22:6	2.0 ± 0.3	4.2 ± 0.2
18:0a/20:4	1.7 ± 0.2	2.8 ± 0.2
18:0a/22:5	1.0 ± 0.3	2.7 ± 0.3
18:0a/22:4	—	0.4 ± 0.1
18:0a/18:2	trace[c]	—
Total	13.3	31.5
18:1a/20:5	1.7 ± 0.2	7.9 ± 0.2
18:1a/22:6	2.2 ± 0.4	6.8 ± 0.2
18:1a/20:4	—	2.8 ± 0.1
18:1a/22:5	1.0 ± 0.1	3.6 ± 0.9
Total	4.9	21.1
16:0a/16:1	1.1 ± 0.1	—
16:0a/18:1 + 18:1a/16:0	13.3 ± 0.9	9.4 ± 0.5
17:0a/16:1	—	trace
17:0a/18:1	0.9 ± 0.2	1.5 ± 0.3
17:0a/20:1	trace	0.2 ± 0.1
18:0a/16:1	0.3 ± 0.1	0.5 ± 0.1
18:0a/18:1 + 16:0a/20:1	21.4 ± 0.7	7.6 ± 0.2
18:0a/20:1	0.4 ± 0.1	0.8 ± 0.1
Total	37.4	20.0
16:0a/14:0	0.4 ± 0.3	—
16:0a/16:0	1.7 ± 0.3	trace
16:0a/18:0 + 18:0a/16:0	0.4 ± 0.2	0.4 ± 0.1
17:0a/16:0	0.7 ± 0.1	1.1 ± 0.1
17:0a/18:0	—	trace
18:0a/18:0	0.7 ± 0.1	0.5 ± 0.1
Total	3.9	2.0
18:1a/16:1	3.1 ± 0.7	—
18:1a/16:1 + 16:0a/16:1	—	5.1 ± 0.2
18:1a/18:1	28.6 ± 1.5	14.8 ± 0.5
18:1a/20:1	2.8 ± 0.1	1.9 ± 0.1
Total	34.5	21.8
Unknowns	5.1 (5)	4.5 (6)

[a]The following fatty acid and dimethylacetal isomers were identified by gas-liquid chromatography: 16:1, n-7 and n-9; 18:1, n-7 and n-9; 18:1a, n-7 and n-9; 20:1, n-7 and n-9; 18:2n-6; 20:4n-6; 20:5n-3; 22:4n-6; 22:5n-3; 22:6n-3. In 1-*O*-alk-1′enyl-2-acyl-glycero-3-phosphoethanolamine (GPE) from both trout and cod it was not possible to resolve 16:0a/22:4n-6 and 18:1a/22:4n-6, while in 1-*O*-alk-1′-enyl-2-acyl-GPE from trout it also proved impossible to satisfactorily resolve 16:0a/22:5n-3 and 18:1a/20:4n-6, and in cod, 18:1a/16:1 and 16:0a/16:1. Values are triplicate determinations of pooled samples from 10 trout and 20 cod brains rounded to the nearest decimal place. The errors are given as ±1 SD.

[b]Not detected.

[c]Trace, < 0.2%.

taining molecular species of 1-*O*-alk-1′-enyl-2-acyl-GPE and of phosphatidylserine were selectively degraded (30), and in human neutrophils 1-*O*-alk-1′-enyl-2-acyl-GPE was the major source of arachidonate liberated when the cells were challenged with ionophore A23187 (31). The large compositional differences between diacyl-GPE and 1-*O*-alk-1′-enyl-2-acyl-GPE in some tissues may thus reflect differences in both the structural and metabolic role of these subclasses.

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Phospholipids in *Drosophila* Heads: Effects of Visual Mutants and Phototransduction Manipulations

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A procedure was developed to label phospholipids in *Drosophila* heads by feeding radioactive phosphate ($^{32}P_i$). High-performance thin-layer chromatography showed label incorporation into various phospholipids. After 24 h of feeding, major phospholipids labeled were phosphatidylethanolamine (PE), 47%; phosphatidylcholine (PC), 24%; and phosphatidylinositol (PI), 12%. *Drosophila* heads have virtually no sphingomyelin as compared with mammalian tissues. Notable label was in ethanolamine plasmalogen, lysophosphatidylethanolamine, lysophosphatidylcholine and lysophosphatidylinositol. Less than 1% of the total label was in phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. Other lipids labeled included phosphatidylserine, phosphatidic acid and some unidentified lipids. A time course (3–36 h) study revealed a gradual decrease in proportion of labeled PI, an increase in proportion of labeled PC and no obvious change in labeled PE. There were no significant differences in phospholipid labeling comparing the *no* receptor *p*otential *(norpA)* visual mutant and wild type under light *vs.* dark conditions. However, overall ^{32}P labeling was higher in the wild type fed in the light as compared to the dark and to *norpA* either in light or dark. This suggests that functional vision facilitates incorporation of label. Differences in phospholipid labeling were observed between young and aged flies, particularly in lysophospholipids and poly-PI, implicating phospholipase A_2 function in recycling. Manipulations such as the *o*uter *r*habdomeres *a*bsent and *ey*es *a*bsent mutants and carotenoid deprivation failed to yield notable differences in phospholipid labeling pattern, suggesting that phospholipids important to vision may constitute only a minor portion of the total labeled pool in the head.
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Photoreceptor membranes in *Drosophila* undergo constant turnover in order to reduce the hazards of light exposure (1–3). Such maintenance involves renewal of the major membrane protein, opsin (4), as well as lipids. In the fly, phospholipids in the photoreceptor membrane are known to play a role in signal transduction (5). In *Limulus,* receptor stimulation of hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) was shown to result in the release of inositol trisphosphate (IP_3) which, in turn, serves as a second messenger for intracellular calcium mobilization (6–9). The *Drosophila* mutant *norpA* has *no* receptor *p*otential because of a defect in phospholipase C (10,11). *NorpA* is a PLC gene which has been cloned (12) and shows homologies to that in bovine brain (13).

Little is known regarding the distribution and metabolic activity of the phospholipids in *Drosophila* and variances in different mutants. This study describes a novel procedure for labeling the membrane phospholipids in the heads of normal, mutant and carotenoid deprived *Drosophila.* An improved high-performance thin-layer chromatography (HPTLC) procedure (14) allowed the analysis of the phospholipids that were labeled. The labeling protocol was used to study the effects of light and dark, mutants and carotenoid deprivation on membrane phospholipids. Brief reports of some of these findings have been published (15–17).

MATERIALS AND METHODS

Animals. Stocks of *Drosophila melanogaster* were raised at room temperature under cyclic lighting on a standard diet adequate for visual photoreceptor development (18). In the medium of agar, brewers yeast, molasses and cornmeal, the most relevant features were yellow cornmeal and a supplement of β-carotene (0.13 mg/mL) as sources of vitamin A. Carotenoid deprivation was achieved using a medium with defined components, Sang's medium (19), and the control here involved supplementing Sang's food with β-carotene at 0.13 mg/mL. Wild type (Oregon-R) and *w*hite *(w)* were used as normal (red-eyed) and white-eyed controls, respectively. Our red-eyed *norpA* was $norpA^{EE5}$, an effective allele (20) originally from Seymour Benzer at the California Institute of Technology (Pasadena, CA). For white-eyed *norpA,* and to provide a comparison allele also known to be effective (3,21), a stock of $norpA^{P24}$//C[1]Dx *y f; cn bw* (*c*in*n*abar *br*ow*n*) was constructed and supplied by Mitchell Dushay of Jeffrey Hall's laboratory at Brandeis University (Waltham, MA). Because only males had the *norpA* genotype, the females being heterozygous, only males were used for the mutant and control *(w)* flies in this experiment. We used the electroretinogram to verify our *norpA* stocks. An allele of *e*yes *a*bsent *(eya)* which lacks the compound eyes but not the simple eyes (ocelli) was obtained from Terry McGuire at Rutgers University (New Brunswick, NJ). The well studied *o*uter *r*habdomeres *a*bsent *(ora)* mutant (22,23) selectively deletes the outer rhabdomeres (R1-6), the predominant photoreceptor type in the compound eye. For *ora,* flies were aged in cyclic lighting one week or more because this manipulation insured a better deletion of the rhabdomeres

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Abbreviations: *cn bw, cinnabar brown*; DG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'* tetraacetic acid; *eya, eyes absent*; GC, gas chromatography; HPTLC, high-performance thin-layer chromatography; IP_3, inositol trisphosphate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol; NL, neutral lipids; *norpA, no receptor potential; ora,* outer *r*habdomeres *a*bsent; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE_{pl}, ethanolamine plasmalogen; P_i, inorganic phosphorus; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PS, phosphatidylserine; TG, triglyceride; TLC, thin-layer chromatography; UN, unknowns; *w, w*hite.

(23). The wild type controls in the *ora* experiments were similarly aged.

^{32}P incorporation and extraction. Flies were transferred into foam stoppered glass vials that contained three circles of filter paper (Whatman No. 3) soaked with 30–50 μCi of ^{32}P-inorganic phosphorus (P_i) in the form of Na_2HPO_4 (New England Nuclear, Wilmington, DE) added to 200 μL of 0.32 M sucrose solution. This amount was adequate for a 30-head sample when flies were fed for 24 h. Flies were fed either in the light or in the dark—dark vials were covered with black tape and light ones with translucent tape. Vials were placed near a fluorescent lamp under a humidified glass dish. Vials were plunged into liquid nitrogen and, when the flies were frozen, vials were shaken and rapped vigorously. By this procedure, heads were well separated from bodies and were sorted quickly with a brush at room temperature. These heads were then put into 1 mL of ice-cold 0.32 M sucrose with 50 mM Tris-HCl buffer (pH 7.4) and ethylenediaminetetraacetic acid (EDTA) (1 mM) and homogenized in a glass homogenizer tube fitted with a motor-driven teflon pestle. Lipids were extracted by adding 4 vol of chloroform/methanol (2:1, vol/vol), to the homogenate, followed by vortexing. After centrifugation at 1500 rpm for 5 min, the organic phase was removed and transferred into an evaporating cup. For more complete extraction of acidic phospholipids, a second extraction was performed with 2 vol of acidic chloroform/methanol/12 N HCl (2:1:0.0125, vol/vol/vol), and the organic phase was neutralized with one drop of 4 N NH_4OH before transferring to the evaporating cup. The extract was evaporated to dryness and resuspended in a small volume of chloroform/methanol (2:1, vol/vol). Aliquots were taken for counting of radioactivity using a Beckman LS5800 liquid scintillation counter (Beckman Instrument, Fullerton, CA).

High-performance thin-layer chromatography (HPTLC). For separation of phospholipids, samples were spotted onto 10 × 10 cm Silica gel 60 HPTLC plates (E. Merck, Darmstadt, Germany) which had previously been dipped in a solution containing 1% potassium oxalate and 2 mM EDTA and diluted with methanol in a ratio of 3:2 (vol/vol). The procedures for separation of phospholipids were described by Sun and Lin (14). Plates were developed in one dimension in a solvent system containing chloroform/methanol/acetone/29% ammonium hydroxide (70:40:10:10, by vol). After development, plates were dried and developed again in the same dimension with a solvent system containing chloroform/methanol/29% ammonium hydroxide/water (72:56:4:12, by vol). The second solvent system was specifically used to move PIP and PIP_2 from the origin. In some experiments, where poly-PI (phosphatidylinositol) separation was not deemed necessary, this solvent system was omitted. After development in the second solvent system, plates were exposed to HCl vapor for 3 min to hydrolyze the alkenyl groups of plasmalogen (24). After drying, plates were turned 90° and developed in a third solvent system containing chloroform/methanol/acetone/acetic acid/0.1 M ammonium acetate (70:30:27.5:2.25:5, by vol). After removal of organic solvent, plates were exposed to iodine vapor for visualization. Furthermore, plates were exposed to Kodak (Rochester, NY) X-omatic AR film for autoradiography of the samples. The phospholipid fractions were scraped into scintillation vials for measurement of radioactivity.

RESULTS

Figure 1 shows a typical autoradiogram and identifies the phospholipids extracted from 75 heads after exposure to $^{32}P_i$ for 26 h. The figure shows incorporation of $^{32}P_i$ into minor as well as major phospholipid components. We have consistently observed a spot to the right side of phosphatidylcholine (PC) on the TLC (thin-layer chromatography) plate (PC_r). Analysis of the fatty acid composition of this lipid fraction indicated a higher content of saturated fatty acids (14:0 and 16:0) and a lower content of 18:1 and 18:2 than in PC. Therefore, PC_r is probably a subspecies of PC. We also have observed two labeled lipid fractions to the right of phosphatidylethanolamine (PE). These spots seem to correspond to phosphatidylglycerol and cardiolipin, but the fractions were not firmly identified. Unlike mammalian tissues, practically no sphingomyelin, cerebrosides or ethanolamine plasmalogen (PE_{pl}) were detected in these samples. On the other hand, notable amounts of lysophospholipids were found.

Data in Figure 2 show the phospholipid labeling profiles in *norpA* and wild type, and compare feeding in light *vs.* dark for 24 h. Results showed no major differences in phospholipid distribution between *norpA* and wild type or between light and dark. The distributions of label in PE and PC, as determined from these and many other experiments, are around 47 and 24%, respectively. PC_r

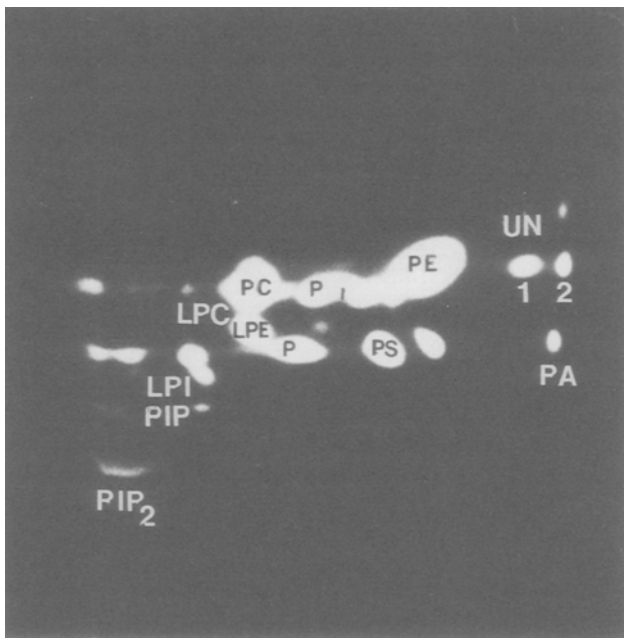

FIG. 1. Autoradiogram of a typical thin-layer chromatography separation of the lipids from *Drosophila melanogaster* heads developed in three solvent systems (ref. 14). The autoradiogram is slightly overexposed to reveal also those phospholipids which are present in small quantities. PIP_2, phosphatidylinositol 4,5-bisphosphate, PIP, phosphatidylinositol 4-phosphate; LPI, lysophosphatidylinositol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; PC_r, spot to the right of phosphatidylcholine: PE, phosphatidylethanolamine; PS, phosphatidylserine; and PA, phosphatidic acid; UN 1 and 2 are unknown fractions also presented in Figures 2 and 4; they may correspond to the cardiolipins.

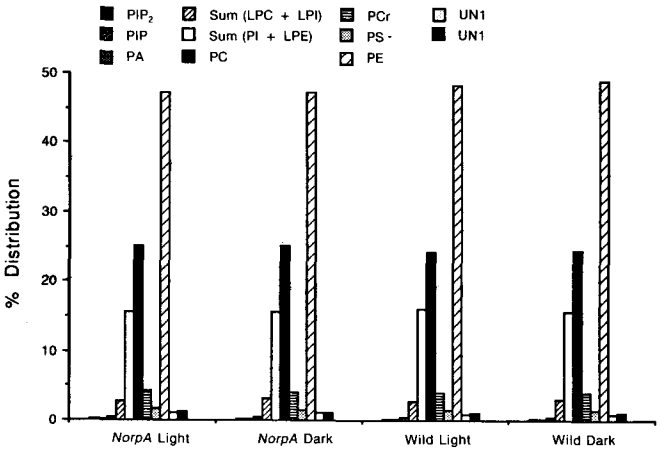

FIG. 2. The percentage distribution of ^{32}P radioactivity in major phospholipids in the heads of the flies studied (*norpA vs.* wild-type, light *vs.* dark reared). Data are pooled from 9 groups of samples (each consisting of 30 heads) in 4 experiments for *norpA* and 7 samples in 3 experiments for wild type. Variations within groups normally do not exceed 5%. Abbreviations as in Figure 1.

comprised 4% of the total radioactivity. In some experiments, the PI spot and the lysoPE (LPE) spot were not clearly separated, and the counts were combined. Nevertheless, on the basis of the plates with good separation, PI was found to comprise 11% and LPE only 2%. Other lysophospholipids, such as lysoPC (LPC) and lysoPI (LPI), comprised 1.6 and 1.4% of total radioactivity, respectively. Other minor phospholipids showing the label are: phosphatidic acid (PA), 0.4%; PIP_2, 0.2%; PIP, 0.2%; phosphatidylserine (PS), 1.6%; unknowns $(UN)_1$, 2.5% and UN_2, 1.1%. Although PS was only labeled sparingly, this phospholipid was a major phospholipid in mass.

We used information from seven experiments to assess incorporation of $^{32}P_i$ into the phospholipids of wild type and *norpA* under light and dark conditions (Fig. 3). Ratios

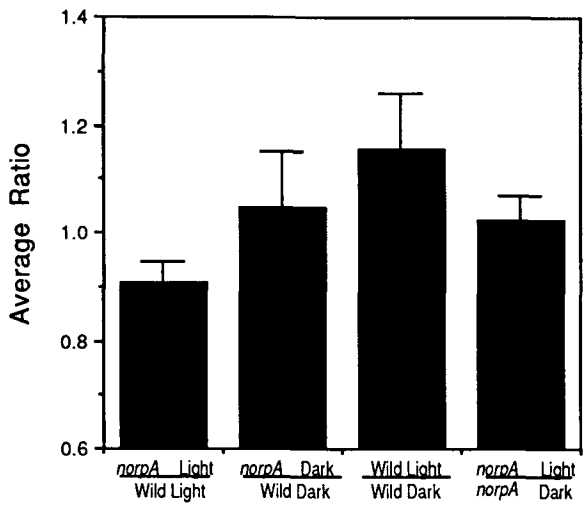

FIG. 3. Overall incorporation of ^{32}P into the phospholipids. Data are based on: (i) the overall counts from aliquots of sample; and (ii) the sum of radioactivity of the lipids on the plates. Data were normalized for the number of fly heads used and the amount of ^{32}P fed. Ratios were calculated using 17 values ± SE except for *norpA* light/*norpA* dark, which was from 19 values ± SE. *NorpA, no* receptor potential.

of about 1 are shown for *norpA*-dark over wild-dark and for *norpA*-light over *norpA*-dark, suggesting that the mutation has no effect in the dark and that the light/dark manipulation has no effect in the mutant. A ratio of about 1.15 was found for wild-light over wild-dark, and the ratio was about 0.9 for *norpA*-light over wild-light. These ratios tend to show that wild type flies have more feeding activity resulting in more labeling in the light, but no dark/light differences are shown for *norpA*, probably because these flies are blind.

When flies were presented with the $^{32}P_i$ for 4 d, rehydrating the filter paper with sucrose but not ^{32}P, the amount of radioactivity incorporated into the phospholipids was similar to that observed after 24-h exposure. However, differences in the phospholipid labeling profile were observed between the 24-h and the 4-d exposure. Data in Table 1 summarize the changes by expressing the ratio between the two data points. With the exception of PC, which did not change as a function of feeding time, substantial deviations from a ratio of 1.0 are evident for most of the lipids. For example, a ratio of less than 1 for PI indicated that the proportion of radioactivity in this phospholipid decreased during 4 d, and ratios greater than 1 for PS, PC_r and lysophospholipids indicate that the proportion of radioactivity of these phospholipids increased during 4 d of feeding. Interestingly, lysophospholipids tended to accumulate more in *norpA* than in wild type irrespective of lighting conditions. In this experiment, only 10 μCi/vial of ^{32}P was given, as it was expected that the 4 times longer feeding would balance the 5 times lower dose. However, incorporation was about 1/4, suggesting that most of the ^{32}P incorporation into phospholipids occurred during the first 24 h. On this basis, we conclude that this experiment simulates the pulse-chase type of study.

To further elaborate on these results, a time course experiment was carried out in which flies were analyzed at 3, 7, 16, 22 and 34 h after ^{32}P feeding. The data in Figure 4 show the distribution of radioactivity among the lipids for wild type (Fig. 4a) and *norpA* (Fig. 4b). In general, the phospholipid labeling patterns in *norpA* and wild type do not differ appreciably, but substantial changes in labeling pattern were observed with respect to time of ^{32}P feeding. For example, there is a time-dependent decrease

TABLE 1

Ratio of Chronic[a] over Acute[b] Incorporations into Selected Phospholipids of *Drosophila* Heads

Lipid[c]	*norpA* light	*norpA* dark	Wild light	Wild dark
PI	0.32575	0.40187	0.35354	0.38940
PS	2.10550	2.37810	3.15360	2.48900
PC	1.19600	1.07600	0.94984	0.92615
PCr	1.47800	1.60700	1.65740	2.25100
Sum LP	4.39600	4.03800	2.84500	3.36500
Sum PIP_2, PIP	1.27600	1.97200	2.20400	1.40000
PA	1.40000	0.89375	0.76000	0.92667

[a] Four-day feeding.

[b] One-day feeding.

[c] PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; LP, lysophospholipids; PIP_2, PI 4,5-bisphosphate; PIP, PI 4-phosphate; PA, phosphatidic acid; *norpA, no* receptor potential.

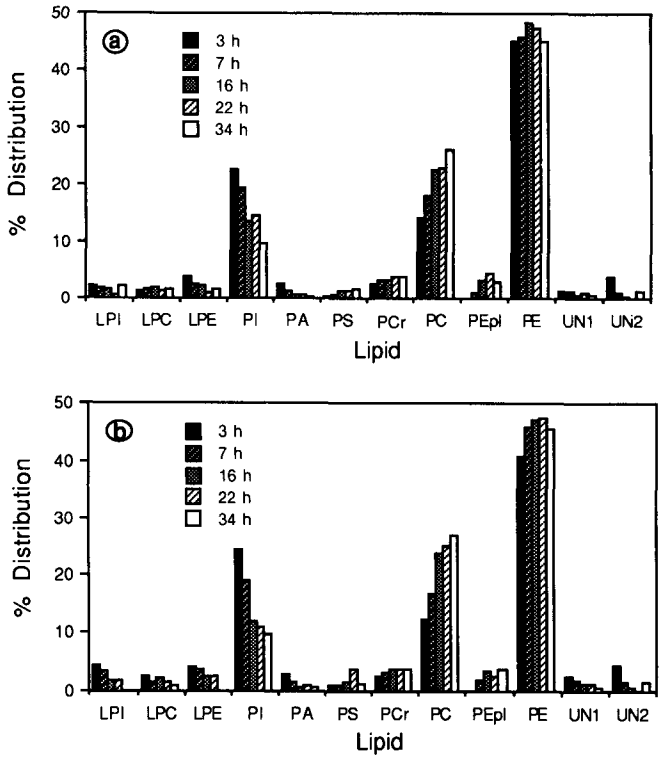

FIG. 4. Distribution of the radioactivity of phospholipids with respect to the duration of ^{32}P exposure: (a) for wild type; (b) for *norpA*. Abbreviations as in Figure 1.

in labeled PI, PA and lysophospholipids, and an increase in labeling of PC, PC_r and PS. Although label was highest in PE, the proportion of label associated with PE did not change greatly with time.

A study was performed to compare the phospholipid labeling pattern in aged *vs.* newly emerged flies. Although no profound changes were observed in the major phospholipids, aging may have increased the labeling of PIP and PIP_2 in wild type, but not in *norpA*, and decreased the labeling of LPC and LPI in all types (Fig. 5).

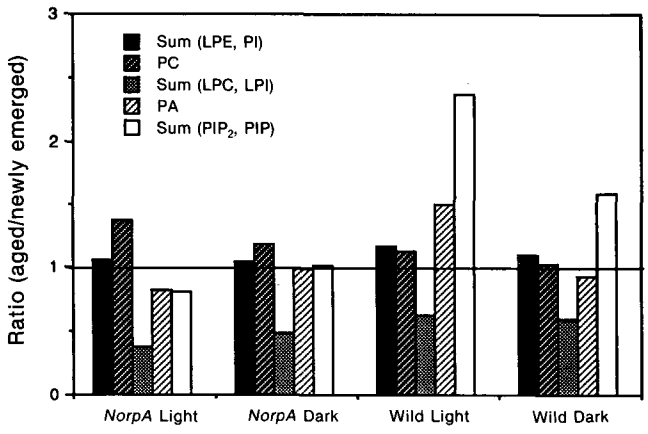

FIG. 5. Ratio of some representative phospholipids for flies aged one week compared with newly emerged flies. The ratio represents the percent distribution of lipids in the aged *vs.* newly emerged flies. Two plates each are pooled from one representative experiment for aged *vs.* newly emerged flies. Abbreviations as in Figure 1.

In pilot experiments, various time intervals were used for dissecting and sorting the heads, and we observed drastic differences in the labeling of lysophospholipids. For all of the data reported above, heads were more rapidly isolated by freezing in liquid nitrogen. When sample preparation was deliberately delayed, apparent lysophospholipid labeling was increased. These results seem to point at the presence of phospholipases in the head which would be responsible for the hydrolysis of the phospholipids.

Using the same feeding paradigm, *i.e.*, 24-h feeding of ^{32}P, labeling of major phospholipids (PA, PS, PI, LPE, PC and PE) was compared for the following groups: (i) wild type controls *vs. eya* (Fig. 6a); (ii) wild type controls *vs. ora* (aged, see methods) (Fig. 6b); (iii) β-carotene supplemented Sang's medium *vs.* Sang's medium which is deficient in carotenoid (Fig. 6c); and (iv) regular food (high carotenoid) controls with Sang's (high carotenoid) flies (Fig. 6d). This last comparison was important because the two diets differed in fatty acid composition (based on gas chromatography (GC) analysis, regular food has 16:0, 74.37%; 18:0, 13.61%; 18:1, 10.06%; and 18:2, 1.96%; whereas Sang's has 16:0, 61.13%; 18:0, 20.49%; 18.1, 18.38%; and 18:2, 0%). The data show that, despite the subtle differences that exist, the overall profiles for ^{32}P incorporation remain largely similar between the various groups.

DISCUSSION

Agonist stimulation of phosphoinositide hydrolysis by PLC is known to give rise to two second messengers, IP_3 and diacylglycerol (DG). The importance of IP_3 on Ca^{++} homeostasis (25) now explains the long realized importance of Ca^{++} on invertebrate photoreceptor function in transduction and/or adaptation. DG activates protein kinase C (PKC), two of which (one specific to the eye) have been cloned in *Drosophila* (26). DG can be converted to PA by DG kinase, an enzyme of interest in visual transduction as it is missing in the *rdgA* (retinal *d*egeneration) mutant (27). In this study, PA is consistently labeled after exposure of flies to ^{32}P, suggestive of its role as an intermediate for phospholipid biosynthesis.

PLC has been shown to play a role in photoreceptor function in *Drosophila* (5). The *norpA* mutant is interesting because it has rhodopsin but lacks the receptor potential (21). It was proposed that this mutant is like dominantly inherited night blindness in humans and the *rd* (retinal *d*ystrophic) chicken (for additional references, see ref. 20). *NorpA* loses rhodopsin as a function of age (3,28). It accumulates zipper-like membranes, suggested as a form of lipid storage abnormality (29). One of the original goals of this line of research was to design experiments to examine the phospholipids in this mutant and to understand how accumulation of lipids may occur during aging. As the data accumulated, it became obvious that it was also necessary to establish a method to label the membrane phospholipids and to present baseline data.

Our experiments revealed for the first time a novel method to label the phospholipids in *Drosophila* heads. By mixing $^{32}P_i$ with the food, effective labeling of the phospholipids in the heads can be achieved within a short time. Examination of the labeling pattern revealed high percentage of label distributed in PE (45%), followed by PC and PI. The ability to incorporate a large amount of

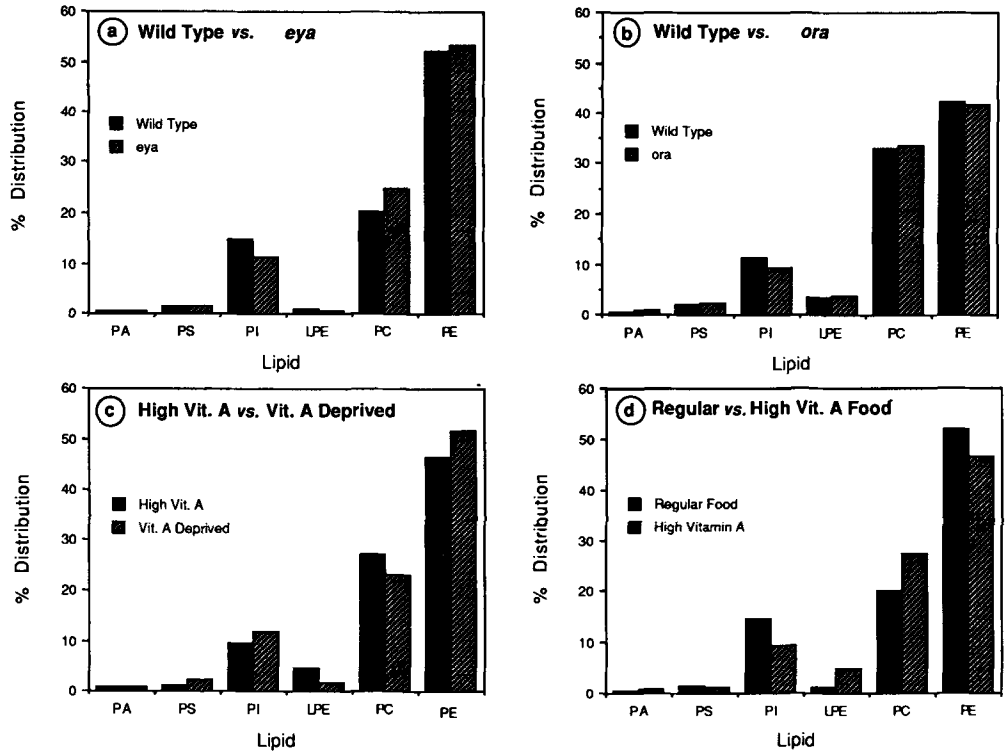

FIG. 6. The percentages of ^{32}P radioactivity in the major phospholipids (PA, PS, PI, LPE, PC and PE normalized to sum to 100%) from the heads of flies upon separation in two solvent systems. Fifty μCi of $^{32}P_i$ per vial was fed and 45 heads of deprived and 45 of the wild type were used. The sample was divided into three aliquots and spotted onto separate plates. The data from these plates were averaged. (a) Wild type *vs. eya*. All flies were newly emerged. (b) Wild type *vs. ora*. All flies were aged 11 d. (c) High β-carotene *vs.* β-carotene deprived flies. Heads were from flies of high β-carotene *vs.* deprived flies reared on Sang's medium. All flies were newly emerged. (d) Regular food *vs.* high Vitamin A food. The regular food control is the same as the *eya* control (Fig. 6a) while the Sang's is the high β-carotene for the vitamin A experiment (Fig. 6c). Abbreviations as in Figure 1. *Eya, eyes absent; ora, outer rhabdomeres absent.*

label into PE in the fly heads is interesting because PC is the predominant lipid in most mammalian tissues. The time course study revealed interesting differences in metabolism between PE *vs.* PI and PC, suggesting a role for PE in the structural make-up of the membrane. Unlike mammalian tissues, phospholipids in *Drosophila* heads are low in sphingomyelins and plasmalogens although the presence of various types of lysophospholipids is apparent. We were disappointed, though not surprised, that only trace amounts of label were incorporated into poly-PI, limiting the usefulness of our labeling procedure for further studies of the metabolism of inositol phospholipids.

Because $^{32}P_i$ is taken up by the flies as food, it is not possible to specifically address the labeling of phospholipids in the retina. In contrast, intracerebral injection of $^{32}P_i$ into mammalian brain can yield effective labeling of the poly-PI (14). Our inability to isolate retina from head, together with the fact that only trace amount of label is in poly-PI, may explain why we did not observe a difference in phospholipid labeling pattern between *norpA* and wild type, although it has clearly been established that the *norpA* mutant lacks PLC (10,11). Also, the *norpA* gene codes for PLC (12), which acts on the poly-PI as well as on PI (30). In the crab, an inositol trisphosphatase terminates the action of IP_3 in mediating visual transduction (31). In bovine brain, there are at least five different types of phospholipase C which are specific to inositol phospholipids (32).

We observed a difference in the overall labeling between *norpA* and wild type, likely attributable to a visually-mediated difference in the feeding behavior. Because *norpA* lacks the photoreceptor potential, these flies did not show a light-mediated increase in feeding. Another interesting observation is the comparison of phospholipid labeling between aged and newly emerged *norpA* and wild type flies. In both dark and light conditions, the aged wild type showed a ratio of greater than 1 for the labeling of poly-PI, whereas the ratios for *norpA* were not changed. The inability to show an age difference in the metabolism of poly-PI in *norpA* may reflect the defective metabolism of poly-PI turnover.

An interesting observation from this study is the presence of a significant amount of lysophospholipids in the heads, suggesting the presence of endogenous phospholipase A_2 (PLA_2) activity. In pilot experiments in which fly heads were not quickly broken off by freezing, we obtained evidence that varying amounts of lysophospholipids were released *post-mortem*. As the proportion of label of these lysophospholipids decreased in the chronic feeding (pulse chase) experiment (Table 1), it is reasonable to conclude that these compounds undergo metabolism and may form an integral part of the phospholipid

turnover cycle. Aging resulted in a decrease in the labeling of LPC and LPI, but there was no effect of mutant condition. We found that there was no effect of light on the appearance of lysophospholipids, though light seems to affect PLA_2 activity in vertebrate rods (33). In other systems, PLA_2 is known to serve as a direct source of arachidonate (34). However, analysis of the fatty acids of the phospholipids revealed no appreciable amount of arachidonic acid in *Drosophila* (data not shown). Zimmerman and Keys (35) characterized an enzymatic system responsible for the removal of LPC and suggested that the lysophospholipids generated by PLA_2 may be involved in the early stages of light-induced damage (35,36). In other studies, PLA_2 was shown to participate in "retailoring" of fatty acids in the 2-position (37). Thus, oxidized polyunsaturated fatty acids can be eliminated from the system through this mechanism. The lysophospholipid-acylCoA acyltransferases are important for recycling polyunsaturated fatty acids to the *sn*-2 position (38).

A comparison of the phospholipid labeling patterns was made among mutants including *norpA, ora* and *eya.* This type of "genetic dissection" is also underway in mouse (*rd* mutant) (39) and dog (*p*rogressive *r*od-*c*one *d*egeneration mutant) (40,41). We also dissected the system with carotenoid deprivation which selectively eliminates rhodopsin (42). This study suggests that the "annular lipids" are important for rhodopsin function (43).

Our results are consistent with the notion that $^{32}P_i$ is incorporated into phospholipids quickly and that some lipids do turnover more rapidly than others. Historically, in other systems, the fast turnover in membrane lipids heralded the discovery of lipid involvement in messenger systems. Our results provide the initial characterization of membrane phospholipids of *Drosophila* heads in wild type and vision mutants. This information will serve as an important base for understanding the mechanism of phototransduction. Future directions are likely to center around the functional role of lipids in visual receptors.

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Stereospecificity of Monoacylglycerol and Diacylglycerol Acyltransferases from Rat Intestine as Determined by Chiral Phase High-Performance Liquid Chromatography

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Using chiral phase high-performance liquid chromatography of diacylglycerols, we have redetermined the ratios of 1,2-/2,3-diacyl-*sn*-glycerols resulting from acylation of 2-monoacylglycerols by membrane bound and solubilized triacylglycerol synthetase of rat intestinal mucosa. With 2-oleoyl[-^{3}H]glycerol as the acyl acceptor and oleoyl-CoA as the acyl donor, 97–98% of the diacylglycerol product was 1,2(2,3)-dioleoyl-*sn*-glycerol, 90% of which was the *sn*-1,2- and 10% the *sn*-2,3-enantiomer. The remaining diacylglycerol (less than 3%) was the *sn*-1,3-isomer. The overall yield of acylation products was 70%, of which 60% were diacylglycerols and 40% triacylglycerols. With 2-oleylglycerol ether as the acyl acceptor and [1-^{14}C]oleoyl-CoA as the acyl donor, 90% of the diradylglycerol was 1-oleoyl-2-oleyl-*sn*-glycerol and 10% was the 2-oleyl-3-oleoyl-*sn*-glycerol. The diradylglycerols made up 96% and the triradylglycerols 4% of the radioactive product. With 1-palmitoyl-*sn*-glycerol as the acyl acceptor and [1-^{14}C]oleoyl-CoA as the acyl donor, the predominant reaction product was 1-palmitoyl-3-oleoyl-*sn*-glycerol. The 3-palmitoyl-*sn*-glycerol was not a suitable acyl acceptor. Both 1,2- and 2,3-diacyl-*sn*-glycerols were substrates for diacylglycerol acyltransferase as neither isomer was favored when 1,2-dioleoyl-*rac*-[2-^{3}H]glycerol was used as the acyl acceptor. There was a marked decrease in the acylation of the 1(3)-oleoyl-2-oleyl-*sn*-glycerol to the 1,3-dioleoyl-2-oleyl-*sn*-glycerol. It is concluded that neither monoacylglycerol nor diacylglycerol acyltransferase exhibit absolute stereospecificity for acylglycerols as fatty acid acceptors.
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During fat absorption, triacylglycerol synthesis in the intestinal mucosa proceeds predominantly *via* acylation of lumenal 2-acylglycerols, although X-1(3)-acylglycerols are also absorbed. The enzymes catalyzing the reesterification of 2-acylglycerols with fatty acids reside together in the endoplasmic reticulum of the enterocyte (1) and are believed to form a triacylglycerol synthetase complex (2) composed of acyl-CoA ligase, monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT). Because the resynthesis of the higher acylglycerols involves acylation of prochiral and chiral esters, the reaction could theoretically proceed either *via* enantiomeric or racemic intermediates. Previous work with whole cells (3), homogenates (3), isolated microsomal membranes (4,5) or purified enzyme preparations (6,7) has led to inconsistent and contradictory conclusions, possibly due to inadequacies in the analytical methodology used for determination of the enantiomeric ratios of synthesized diacylglycerols.

In the present study we have taken advantage of the discovery of a chiral phase high-performance liquid chromatography (HPLC) method for the separation of enantiomeric diacylglycerols (8) and of its adoption for natural diacylglycerol analysis (9). This approach avoids the uncertainties of the past and provides direct and unambiguous information with regards to separation, identification and quantitation of the acylglycerol acylation intermediates, as well as the initial substrates and final products.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleoyl-CoA (specific activity 52 mCi/mmol) and Aquasol were purchased from New England Nuclear/Dupont Canada Inc. (Lachine, Quebec, Canada). [2-^{3}H]Glycerol trioleate (specific activity 2 Ci/mmol) was obtained from ICN Biochemicals Canada Ltd. (Montreal, Quebec, Canada). Oleoyl-CoA, trioleoylglycerol, oleic anhydride and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was from Bio-Rad Laboratories Canada Ltd. (Mississauga, Ontario, Canada). Silica gel H (Merck 60 H) and G (Merck 60 G) were obtained from Terochem Laboratories Ltd. (Mississauga, Ontario, Canada). Silica gel GF thin-layer chromatography (TLC) plates (20 × 20 cm, 0.25-mm thick) were purchased from Analtech Inc. (Newark, DE). 2-Oleylglycerol was from Serdary Research Laboratories (London, Ontario, Canada). The 1- and 3-palmitoyl-*sn*-glycerols were gifts from Dr. D. Buchnea (University of Toronto). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

Preparation of acyl acceptor substrates. 2-Oleoyl-[2-^{3}H]glycerol (specific activity 3.3 mCi/mmol) was prepared by digestion of [2-^{3}H]glycerol trioleate with porcine pancreatic lipase (10). The 1,2(2,3)-dioleoyl-*sn*-[2-^{3}H]-glycerol (specific activity 0.25 mCi/mmol) was prepared by Grignard degradation of [2-^{3}H]glycerol trioleate (10). 1,3-Dioleoyl-2-oleyl-*sn*-glycerol was prepared by chemical synthesis from 2-oleylglycerol and oleic anhydride as follows: four mg 4-dimethylaminopyridine and 90 μmol oleic anhydride were added to 90 μmol of 2-oleylglycerol in 150 μL dry toluene. After 5 min at room temperature the reaction mixture was dried under nitrogen, and the 1,3-dioleoyl-2-oleyl-*sn*-glycerol was recovered following

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Abbreviations: BCA, bicinchonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; CI, chemical ionization; DG, diradylglycerol; DGAT, diacylglycerol acyltransferase; DNPU, dinitrophenylurethane; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate (sodium salt); GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MG, monoradylglycerol; MGAT, monoacylglycerol acyltransferase; PMSF, phenylmethylsulfonyl fluoride; TG, triradylglycerol; TLC, thin-layer chromatography; Tris, tris[hydroxymethyl]aminomethane; UV, ultraviolet.

TLC on silica gel H developed in heptane/diisopropyl ether/acetic acid (60:40:4, vol/vol/vol). X-1(3)-oleoyl-2-oleylglycerol was prepared by digestion of the triradylglycerol with pancreatic lipase (10). The purities and concentrations of the radiolabeled and unlabeled substrates were assessed by boric acid TLC and by gas-liquid chromatography (GLC) on a polar capillary column.

Animals. Male Wistar rats weighing 250–300 g were purchased from Charles River Canada Inc. (La Salle, Quebec, Canada) and were housed for 1–3 wk with free access to Purina Rodent Chow and water before being used in these studies.

Preparation of subcellular fractions. The animals were anesthetized with diethyl ether and exsanguinated *via* their abdominal aortae. The mucosal scrapings of the upper two-thirds of the small intestine were isolated as described by Hoffman and Kuksis (11). Supernatant devoid of brush border membranes, obtained after homogenization of the scrapings and low-speed centrifugation (12), was spun at 25,000 × *g* for 10 min, and the postmitochondrial supernatant was further centrifuged at 106,000 × *g* for 60 min to pellet microsomal membranes. Microsomes were washed with 50 mM tris[hydroxymethyl]aminomethane (Tris)-HCl (pH 7.4), 0.5 M KCl, 1 mM ethylenediaminetetraacetate (EDTA), 2 mM DTT and sedimented by re-centrifugation at 106,000 × *g* for 60 min. All steps were carried out at 0–4°C. Washed microsomes were suspended in 50 mM K_2HPO_4 (pH 7.4), 1 mM EDTA, 1 mM DTT and 75 μg/mL PMSF to give a final concentration of protein between 7–8 mg/mL. Protein content was determined by bicinchonic acid (BCA) assay (Pierce Chemical Co., Rockford, IL.)

Preparation of triacylglycerol synthetase. Microsomes were solubilized with 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonate (CHAPS) for 30 min on ice, and the solubilized extract was loaded on a Cibacron Blue 3GA agarose column (1 × 5 cm) preequilibrated with 20 mM K_2HPO_4 (pH 7.4), 2 mM DTT and 0.5% CHAPS (Buffer A). The column was washed with Buffer A, followed by Buffer A containing 10 mM ATP, and the triacylglycerol synthetase activity was subsequently eluted with Buffer A containing 0.8 M NaCl (13).

Enzyme assays. The activities of MGAT and DGAT were determined using acetone dispersed acylglycerols (14). The enzyme fractions (20–25 μg protein) were incubated at 37°C for 10 min in 0.5 mL of 20 mM K_2HPO_4, pH 7.4, 1 mM DTT, 1 mM EDTA containing [1-^{14}C]oleoyl-CoA (30 μM), and various monoradylglycerol isomers (60 μM) or 1,2(2,3)-dioleoyl-*sn*-glycerol (250 μM) supplied in acetone (final conc. 2.5%). The reaction was stopped by addition of 4 mL of $CHCl_3/CH_3OH$ (2:1, vol/vol) and lipids were analyzed as described by O'Doherty *et al.* (15). The lipid extracts were separated by TLC on silica gel H employing heptane/diisopropyl ether/acetic acid (60:40:4, vol/vol/vol) as the mobile phase, visualized with iodine vapor, and bands corresponding to 1,2(2,3)-diacyl-*sn*-glycerol, X-1(3)-diacylglycerol and triacylglycerol were scraped off and counted for radioactivity.

Stereospecific analysis. Following enzymatic assay, the 1,2(2,3)-diradyl-*sn*-glycerols formed were separated on boric acid impregnated TLC (silica gel G) plates using chloroform/acetone (96:4, vol/vol) as the mobile phase. The recovered 1,2(2,3)-diradyl-*sn*-glycerols were converted to the corresponding dinitrophenylurethane (DNPU) derivatives according to the procedure of Takagi and Itabashi (8). The crude urethane derivatives were purified by TLC (silica gel GF) using hexane/ethylene dichloride/ethanol (40:10:3, vol/vol/vol). The DNPU derivatives of the enantiomeric diradylglycerols were resolved by HPLC as previously described (8) using *N*-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine as the chiral phase and hexane/ethylene dichloride/ethanol (150:20:1, vol/vol/vol) as the mobile phase. For chiral phase liquid chromatography/mass spectrometry (LC/MS) (Hewlett-Packard 5985B quadrupole mass spectrometer combined with a Hewlett-Packard direct liquid inlet interface, Palo Alto, CA), the original mobile phase was replaced with isooctane/*t*-butyl methyl ether/isopropyl alcohol/acetonitrile (80:10:5:5, vol/vol/vol) containing 1% ethylene dichloride to enhance the negative ion response (16).

RESULTS

Overall utilization of substrate. Table 1 compares the overall synthesis of diradyl- (DG) and triradylglycerols (TG) from various monoradylglycerols (MG) and radiolabeled oleoyl-CoA by the microsomes of rat intestinal mucosa. Starting with 2-oleoylglycerol (60 μM) and 25 μg microsomal protein, 70% of the 2-acylglycerol was incorporated into higher acylglycerols within a 10-min incubation (specific activity 23 ± 4 nmol/mg protein/min). The major acylation products were 1,2(2,3)-dioleoyl-*sn*-glycerols (61.6%) and trioleoylglycerols (35.4%). About 3% of the total was recovered as X-1,3-dioleoylglycerols, which apparently arose from acyl migration during the experimental manipulations.

With 2-oleylglycerol ether (60 μM) as the acyl acceptor and 25 μg microsomal protein, the acylation proceeded at about 80% of the rate seen for 2-oleoylglycerol ester. The main product (96.9%) was 1(3)-oleoyl-2-oleyl-*sn*-glycerol, with only about 3% of the total acylation product being recovered as the 1,3-dioleoyl-2-oleyl-*sn*-glycerol.

The 1-palmitoyl-*sn*-glycerol was utilized even less effectively (34.6% of the acylation rate observed for 2-oleoylglycerol). The major product (81.8%) was 1-palmitoyl-3-oleoyl-*sn*-glycerol with a smaller proportion (18.2%) of 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol. Since X-1,3-diacylglycerols are not acylated at the 2-position, the formation of the triacylglycerols (4.9% of the final product) must be attributed to partial isomerization of the 1-palmitoyl-*sn*-glycerol to 2-palmitoylglycerol, formation of 1,2(2,3)-

TABLE 1

Synthesis of Di- and Triradylglycerols from Monoradylglycerols and [1-^{14}C]Oleoyl-CoA by Microsomes from Rat Intestinal Mucosa[a]

Substrate	Percentage of total product synthesized			Relative activity (%)
	1,2(2,3) DG	1,3 DG	TG	
2-18:1-*sn*-glycerol	61.6	3.0	35.4	100.0
2-18:1-*sn*-glycerol ether	96.9	—[b]	3.1	80.4
1-16:0-*sn*-glycerol	13.3	81.8	4.9	34.6
3-16:0-*sn*-glycerol	58.1	17.4	24.4	0.4

[a] Values are averages of three independent experiments performed in duplicates.

[b] Not found.

palmitoyl-*sn*-glycerols and their further acylation to 1,3-dioleoyl-2-palmitoyl-*sn*-glycerols. Under the same conditions, 3-palmitoyl-*sn*-glycerol was not acylated to the *sn*-1,3-isomer.

Table 2 shows the results obtained with the triacylglycerol synthetase complex purified from rat intestinal microsomes (MGAT specific activity 165 ± 23 nmol/mg protein/min).

Enantiomeric specificity. For this analysis, which represents mass ratios of formed diacylglycerols, lipid extracts from five incubations were combined and applied to a TLC plate in the absence of lipid carriers. The 1,2(2,3)-diacyl-*sn*-glycerols were eluted from the gel and converted to the DNPU derivatives. The incubation blank contained 1,2(2,3)-diacyl-*sn*-glycerol DNPU derivatives from five incubations performed in the absence of exogenous monoradylglycerols and oleoyl-CoA, and represent endogenous levels of microsomal diacylglycerols. Figure 1 shows the ultraviolet (UV) absorption profile of the DNPU derivatives of standard diacylglycerols (A) and of the diacylglycerols (B) recovered from incubation of the microsomes with radiolabeled 2-oleoylglycerol and oleoyl-CoA. The incubation products are seen to consist largely of the 1,2-diacyl-*sn*-glycerols with smaller, but clearly recognizable amounts, of the *sn*-2,3-enantiomers. The incubation blank (C) contains UV absorbing peaks that coincide with the 1,2-diacyl-*sn*-glycerol standards of other common diacylglycerol species. In parallel experiments carried out to assess the radioactivity of the diacylglycerol enantiomers formed by microsomal MGAT, the peaks corresponding to the 1,2- and 2,3-diacyl-*sn*-glycerols were collected and rechromatographed under identical conditions (Fig. 2). It can be seen that the collected *sn*-1,2-enantiomer does not contain any *sn*-2,3-isomer (B), whereas the *sn*-2,3-isomer is devoid of the *sn*-1,2-enantiomer (C). Similar enantiomeric separations were obtained for the acylation products of the 2-oleylglycerol ether, which, however, eluted earlier than the corresponding diacylglycerols under the same chiral phase HPLC conditions (Fig. 3). The isolation of these acylalkylglycerol peaks required co-chromatography with the corresponding non-radioactive 1-oleoyl-2-oleyl-*rac*-glycerols.

Table 3 shows that the 2-oleoylglycerol and its ether analog were preferentially esterified by the microsomal MGAT at the *sn*-1-position, giving an *sn*-1-/*sn*-3-acylation ratio of 9. Similar values were obtained for the acylation products of the solubilized triacylglycerol synthetase (Table 4). The radioactivity values are quite similar to those obtained by mass analysis (Figs. 1B and 2A).

The identity of the HPLC peaks was confirmed by chiral phase LC/MS (Fig. 4). The total negative ion current profiles of the incubation product (A) and standard (B) are identical to those of the UV profiles. The bottom part of Figure 4 (C and D) shows the full mass spectra recorded for the DNPU derivatives of the 1,2- and 2,3-dioleoyl-*sn*-glycerols obtained from acylation of 2-oleoylglycerol with oleoyl-CoA in the presence of microsomal MGAT. The 1,2- and 2,3-dioleoyl-*sn*-glycerols gave the $[M]^-$ molecular

TABLE 2

Synthesis of Di- and Triradylglycerols from Monoradylglycerols and [1-^{14}C]Oleoyl-CoA by Solubilized Synthetase from Rat Intestinal Mucosa[a]

Substrate	Percentage of total product synthesized			Relative activity (%)
	1,2(2,3) DG	1,3 DG	TG	
2-18:1*sn*-glycerol	71.2	2.9	25.8	100.0
2-18:1-*sn*-glycerol ether	98.2	—[b]	1.8	77.4
1-16:0-*sn*-glycerol	8.6	87.2	4.3	33.6
3-16:0-*sn*-glycerol	46.3	21.6	32.1	0.6

[a]Values are averages of three independent experiments performed in duplicates.

[b]Not found.

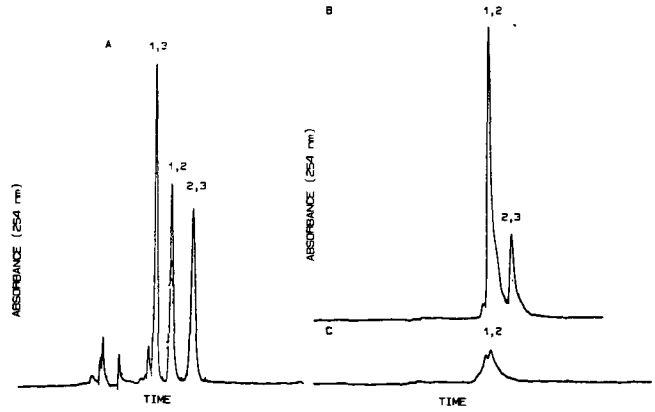

FIG. 1. Ultraviolet absorption profile of the 3,5-dinitrophenylurethane derivatives of standard diacylglycerols (A), of diacylglycerols recovered from incubation of the microsomes with radiolabeled 2-oleoylglycerol and oleoyl-CoA (B), and of endogenous diacylglycerols (C).

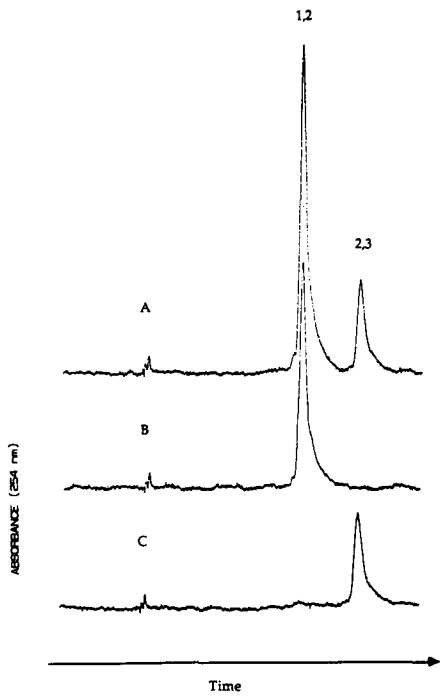

FIG. 2. Ultraviolet absorption profile of the 3,5-dinitrophenylurethane derivatives of diacylglycerols recovered from incubation of the microsomes with radiolabeled 2-oleoylglycerol and oleoyl-CoA (A), and of rechromatographed peaks corresponding to the *sn*-1,2-enantiomer (B) and *sn*-2,3-enantiomer (C).

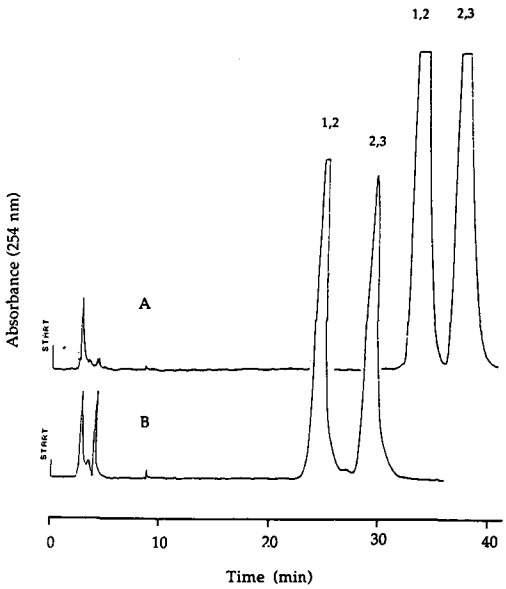

FIG. 3. Ultraviolet absorption profile of the 3,5-dinitrophenylurethane derivatives of diradylglycerols. (A), 1,2(2,3)-dioleoyl-*sn*-glycerol; (B), 1(3)-oleoyl-2-oleyl-*sn*-glycerol.

TABLE 3

Enantiomeric Ratios of 1,2- and 2,3-Diradyl-*sn*-glycerol (DG) Products Synthesized by Microsomal Monoacylglycerol Acyltransferase from Rat Intestinal Mucosa

Substrate	1,2-DG (% total cpm)	2,3-DG (% total cpm)
2-18:1-*sn*-glycerol	90 ± 3 (6)[a]	10 ± 3 (6)
2-18:1-*sn*-glycerol ether	90 ± 1 (3)	10 ± 1 (3)

[a]Numbers in parentheses refer to number of independent determinations performed in duplicates.

TABLE 4

Enantiomeric Ratios of 1,2- and 2,3-Diradyl-*sn*-glycerol (DG) Products Synthesized by Solubilized Triacylglycerol Synthetase from Rat Intestinal Mucosa

Substrate	1,2-DG (% total cpm)	2,3-DG (% total cpm)
2-18:1-*sn*-glycerol	88 ± 2 (4)[a]	12 ± 2 (4)
2-18:1-*sn*-glycerol ether	91 ± 1 (2)	9 ± 1 (2)

[a]Numbers in parentheses refer to number of independent determinations performed in duplicates.

ion (*m/z* 829) and a characteristic $[M - DNPU + 35]^-$ fragment ion (*m/z* 655). This ion corresponds to the addition of a chlorine group to the dioleoylglycerol molecule. The negative chemical ionization (CI) was found to be about 100 times more sensitive than the positive CI current for these compounds under the chiral phase LC/MS conditions (16).

Utilization of the diacylglycerol intermediates for triacylglycerol synthesis was also examined by incubating

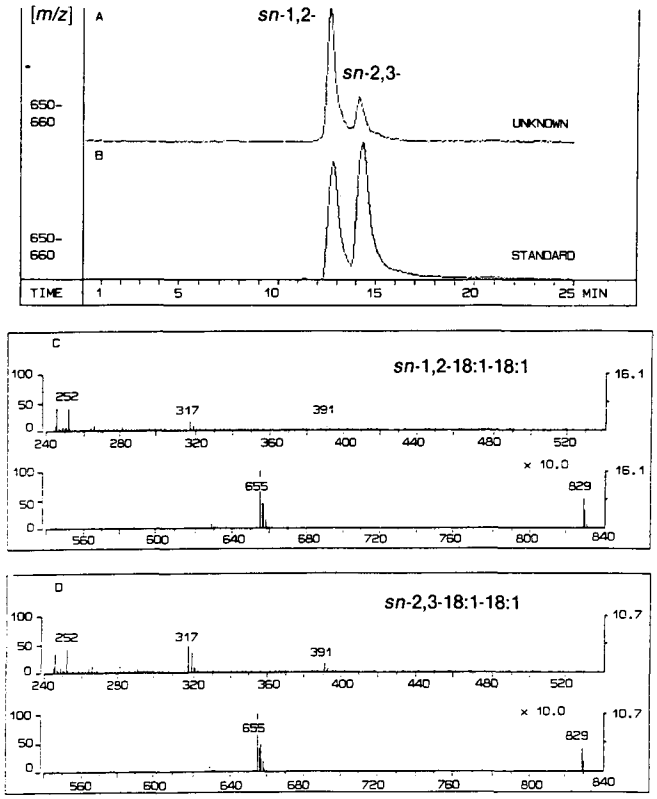

FIG. 4. Chiral phase liquid chromatography/mass spectrometry. The total negative ion current profiles of the incubation product (A) and standard (B). (C) and (D), full spectra recorded for the 1,2- and 2,3-dioleoyl-*sn*-glycerol dinitrophenylurethane derivatives obtained by acylation of 2-oleoylglycerol with oleoyl-CoA and microsomal monoacylglycerol acyltransferase.

microsomes with racemic 1,2(2,3)-dioleoyl-*sn*-[2-^{3}H]-glycerol and oleoyl-CoA. Some 25–30% of the radiolabeled substrate was converted to triacylglycerols (30–40 nmoles). The substrate remaining after incubation was subjected again to chiral HPLC, and the residual *sn*-1,2- and *sn*-2,3-enantiomers resolved and the peaks collected for assay of radioactivity. There was no change in the relative proportions of the mass (Fig. 5) or in the radioactivity in the two enantiomers (Table 5).

DISCUSSION

The present study clearly demonstrated that membrane-bound, as well as solubilized, triacylglycerol synthetase of rat intestinal mucosa is capable of acylating both *sn*-1- and *sn*-3-positions of the 2-acylglycerols, although the *sn*-1-position is preferred. The *sn*-2-position of the 1- or 3-acyl-*sn*-glycerols or X-1,3-diacylglycerols is not acylated. The 1-acyl-*sn*-glycerol yielded exclusively 1,3-diacyl-*sn*-glycerol, and 3-acyl-*sn*-glycerol was not acylated. The above results, which were obtained with substrates and products of known enantiomeric composition and purity, allow critical comparisons with previous results. Thus, the studies confirmed the positional requirements established previously for rat intestine (17), which converts 2-acylglycerols to X-1,2-diacylglycerols and triacylglycerols, and 1-acyl-*sn*-glycerols to 1,3-diacyl-*sn*-glycerols, whereas 3-acyl-*sn*-glycerols are not acylated. Furthermore, the

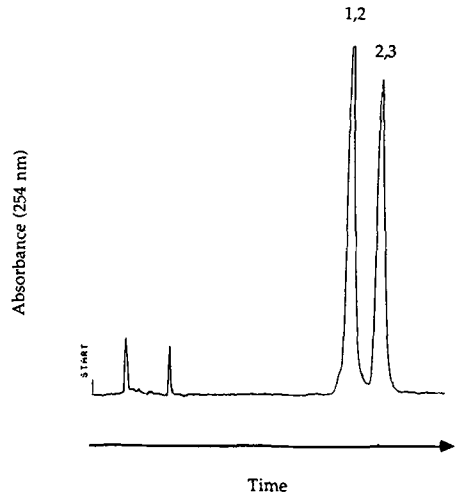

FIG. 5. Ultraviolet absorption profile of the 3,5-dinitrophenylurethane derivatives of the residual 1,2(2,3)-dioleoyl-*sn*-[2-^{3}H]glycerol after incubation of microsomal diacylglycerol acyltransferase with the radiolabeled diacylglycerol and oleoyl-CoA.

TABLE 5

Utilization of 1,2(2,3)-Dioleoyl-*sn*-glycerol (DG) by Microsomal Diacylglycerol Acyltransferase from Rat Intestinal Mucosa

Substrate	1,2-DG (% total cpm)	2,3-DG (% total cpm)
1,2(2,3)-18:1-*sn*-glycerol	53 ± 2 (2)[a]	47 ± 2 (2)

[a]Numbers in parentheses refer to number of independent determinations performed in duplicates.

present study showed that X-1,3-diacylglycerols and X-1-acyl-2-alkylglycerols are not suitable substrates for DGAT. Previous work (17) had shown that DGAT of hamster microsomes utilizes X-1,3-diacylglycerols as well as acylalkylglycerols for triacylglycerol biosynthesis. However, more recent work has shown that the hamster also utilizes the 2-acylglycerols at similar rates as compared to the rat (18). Our microsomal preparations yielded closely similar MGAT specific activities (23 ± 4 nmol/mg protein/min) to those reported by Coleman and Haynes (14) in the absence of activating phospholipids and albumin (28–45 nmol/mg protein/min). Acylation of X-1-acylglycerols yields X-1,3-diacylglycerols which are not esterified further. The earlier studies (17,19) apparently did not eliminate all the uncertainties about the identity and composition of either substrates or products. The present results also differ from the findings in chicken embryos, where 1-oleoylglycerol and 2- and X-1-oleylglycerols and their amide analogs were much less efficiently utilized when compared to 2-oleoylglycerol (19). However, in all instances, a lower activity with 2-alkyl than found with 2-acylglycerol was observed in the present study, as reported previously (17,19).

The acylation of 2-acylglycerol yielded 90% *sn*-1,2- and 10% *sn*-2,3-enantiomers. The results obtained with solubilized MGAT indicate that the 2,3-diacyl-*sn*-glycerol formation cannot be attributed to the slower reaction rate of the microsomal preparations. A similar enantiomer proportion was observed for the acylation of 2-alkylglycerol, although the overall yield of the diacylglycerols was only about 80% of that obtained for the 2-acylglycerol. Furthermore, the 1-acyl-2-alkyl-*sn*-glycerol was only partly converted to the 1,3-diacyl-2-alkyl-*sn*-glycerol, which indicated that the DGAT discriminated more against the 2-alkylglycerol derivatives than did MGAT. The 90% yield of 1,2-diacyl-*sn*-glycerols is similar to the 85–90% originally reported by Johnston *et al.* (20), O'Doherty and Kuksis (4) and, more recently, by Coleman *et al.* (5), but the conclusions differed. Whereas Johnston *et al.* (20) and Coleman *et al.* (5) felt that the enzyme was completely stereospecific, O'Doherty and Kuksis (4) allowed for the possibility that the *sn*-2,3-enantiomers recovered were also products of direct acylation of 2-monoacylglycerols. All previous methods used for enantiomeric determinations employed indirect procedures that required 100% completion of enzymatic conversions of 1,2-diacyl-*sn*-glycerols to phosphatidic acid by diacylglycerol kinase (5) or hydrolysis of 1,2-diacyl-*sn*-glycerophosphophenols by phospholipase A_2 (4). The present study established by direct and unambiguous analysis that about 10% 2,3-diacyl-*sn*-glycerols occur as intermediates during triacylglycerol synthesis from 2-acylglycerols and 2-alkylglycerols. This is also the first instance where both products of enzymatic reaction have been directly measured, rather than estimated by difference from incomplete or partial reactions. The present finding is similar to the 15–19% 2,3-diacyl-*sn*-glycerol yield reported (4) for rat intestinal microsomes. The somewhat higher proportions of 2,3-diacyl-*sn*-glycerols recovered during biosynthesis in isolated cells and mucosal homogenates (3) also may have reflected partial lipolysis of the radioactive triacylglycerols by the endogenous lipases which participate in the lipolysis/reacylation cycle during lipoprotein secretion (21–23). The much higher yield (40%) of 2,3-diacyl-*sn*-glycerols obtained with the 37 kDa polypeptide of MGAT (7) was not confirmed in the present study. The acylation of 2-acylglycerols to 2,3-diacyl-*sn*-glycerols is supported by the isolation of 2-alkyl-3-acyl-*sn*-glycerols during the acylation of 2-alkylglycerols. Because the 2-alkylglycerol is not subject to acyl migration, the recovery of 2-alkyl-3-acyl-*sn*-glycerols cannot be attributed to X-1,3-alkylglycerols.

Finally, this study demonstrated that both 1,2- and 2,3-diacyl-*sn*-glycerols are acylated to triacylglycerols at comparable rates, which would explain why 2,3-diacyl-*sn*-glycerols did not accumulate during the acylation of 2-acylglycerols to triacylglycerols. Direct acylation of enantiomeric diacylglycerols was recognized in the early work of Weiss *et al.* (24), who noted greater than 50% utilization of 1,2-diacyl-*rac*-glycerols by chicken liver particles. As the acylation of 1,2(2,3)-alkylacyl-*sn*-glycerols to alkyldiacylglycerols is very slow, it was not possible to use this enantiomer mixture to confirm the utilization of the racemic diradylglycerols by DGAT. The present findings and the proposed interpretation of the data reconcile essentially all previous experimental findings and corrects earlier conclusions about the stereochemical course of acylation of 2-acylglycerols to triacylglycerols by rat intestinal mucosa.

ACKNOWLEDGMENTS

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Structural Analysis of Oxidation Products of Urofuran Acid by Hypochlorous Acid

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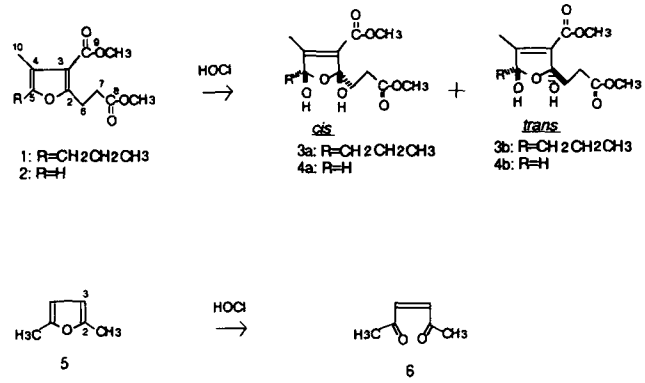
The oxidation of urofuran acid derivatives (1–2) by hypochlorous acid (HOCl) was investigated with the goal to possibly simplify the detection of their metabolites in biological materials. The oxidation products of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (1) were obtained as an isomeric mixture and confirmed to exist as *cis* (3a) and *trans* (3b) isomers, based on their ^{13}C nuclear magnetic resonance (NMR) spectra. Similarly, the products of 5-H substituted acid 2 obtained by oxidation with HOCl were identified as 4a and 4b by ^{13}C and ^{1}H NMR which indicated the presence of *cis* and *trans* hemiacetal hydrogens at C-5 in a ratio of 2.11:1. The oxidation was found to proceed in a manner different from that of the F-acid, because of the presence of the electron withdrawing $COOCH_3$ group at C-3 which favored the nucleophilic attack on the carbonyl group to afford *cis*- and *trans*-2,5-dihydroxy-2,5-dihydrofurans (3a–b, 4a–b).
***Lipids* 28, 35–41 (1993).**

3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid (1) (CMPF), known as urofuran acid, is found in human urine (1) and is retained in the blood of patients with chronic renal failure (2). CMPF inhibits the binding of methyl red to human albumin (3) and the binding of phenytoin and tryptophan to plasma protein (4). Although the 5-pentyl urofuran acid is known to be derived from 9-(3,4-dimethyl-5-pentyl-2-furyl)nonanoic acid (F-acid) present in food (5,6), the metabolic sequence leading to CMPF has not been fully elucidated.

It has been reported recently that various furan derivatives can be oxidized chemically or enzymatically. For example, 2,5-diphenylfuran can be converted to *cis*-1,2-dibenzoylethylene by hypochlorous acid (HOCl) *via* the intermediate 2-chloro-5-hydroxy-2,5-diphenyl-2,5-dihydrofuran (7), and F-acid, when treated with hydrogenperoxide/sodium hypochlorite, leads to methyl 11,12-dimethyl-10,13-dioxo-11-octadecenoate (8), which can also be obtained as an autoxidation product (9) or as an enzymatic oxidation product (10,11). These reports prompted us to investigate the oxidation of CMPF dimethyl ester (1) with HOCl with the goal to possibly simplify the detection of CMPF metabolites in biological materials.

In the present study, we found that the oxidation of 1 proceeded in a manner different from that of F-acid to afford an isomeric mixture of *cis*- and *trans*-2,5-dihydroxy-2,5-dihydrofurans (3a–b), which have not previously been described. The structural identification was confirmed by *spectral data, including* ^{13}C and ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (MS). To verify the *cis/trans* stereochemistry, the CMPF analogue (2), bearing a hydrogen substituent at C-5, was prepared and also subjected to HOCl oxidation under similar conditions. Based on ^{1}H NMR spectral analysis, the structure of the oxidation products (4a–b) was unequivocally confirmed to be the *cis* and *trans* hemiacetals.

In addition, an interesting effect of the electron withdrawing group $COOCH_3$ at C-3 was observed, and the results were compared with the oxidation of 2,5-dimethylfuran (5). The structural formulas and reaction sequences are given in Scheme 1.



SCHEME 1

RESULTS

Characterization of the oxidation products of 1 *by high-performance liquid chromatography (HPLC).* Figures 1A and 1B show the elution profiles of 1 and those of the reaction mixture obtained by oxidation of 1 with HOCl. Peaks 1 and 2 represent the oxidation products with retention times of 4.1 min and 4.6 min. We separated the effluents corresponding to peaks 1 and 2; however, the component ratios changed progressively with time (Fig. 1C). This pattern of behavior strongly suggested that the oxidation products existed as an equilibrium mixture of two tautomers.

Characterization of the oxidation products of 1 *by silica gel chromatography.* The oxidation products (peaks 1 and 2) were fractionated by silica gel column chromatography. The products were eluted with ethyl acetate/hexane (7:3, vol/vol) to give ten fractions. The fractions were analyzed by thin-layer chromatography (TLC) as shown in Figure 2. Fraction 5, combined fractions 6–8, and combined fractions 9–14 were further analyzed. Figures 3A, 3B and 3C

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Abbreviations: CI-MS, chemical ionization mass spectrometry; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (1); COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarization transfer; EI-MS, electron impact ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

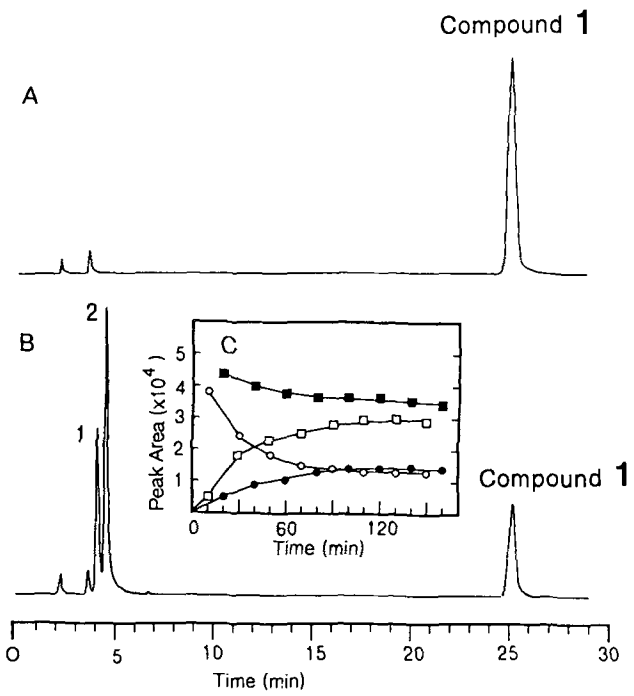


FIG. 1. Elution profiles in high-performance liquid chromatography: (A) 1; (B) reaction mixture injected 30 s after mixing 1 and NaOCl solution; (C) time courses of tautomerism between peak 1 and peak 2: ○, peak 1; □, peak 2 produced from peak 1 (○); ■, peak 2; ●, peak 1 produced from peak 2 (■).

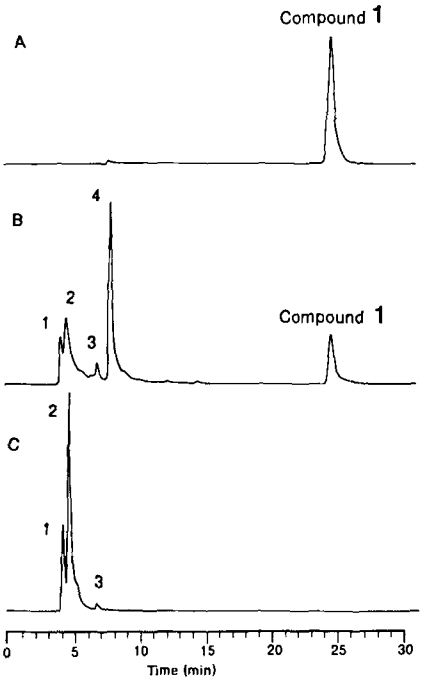


FIG. 3. High-performance liquid chromatography profiles of fractions separated by column chromatography: (A) Fraction 5; (B) Fractions 6–8; (C) Fractions 9–14.

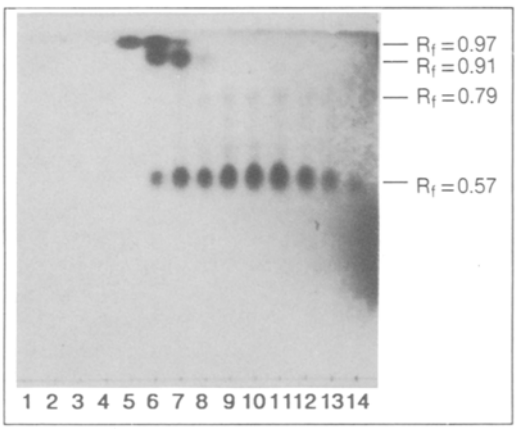


FIG. 2. Thin-layer chromatography of oxidation products.

show the HPLC profiles of the fractions 5, 6–8 and 9–14, respectively. Fraction 5 was identified as 1 based on R_f value and electron impact ionization mass spectrum (EI-MS). Fractions 9–14 (Fig. 3C) consisted mainly of peaks 1 and 2, which are also seen in Figure 1B.

Structural analysis of the oxidation products (**3a–b** *and* **4a–b**). Although our efforts to isolate the two oxidation products in pure form were unsuccessful, structural assignments were carried out as follows. Fast atom bombardment mass spectrum (FAB-MS) showed an $[M - H]^-$ ion peak at *m/z* 301. This fragment indicated a molecular weight of 302, corresponding to 1 plus two hydroxy groups. The ^1H NMR spectrum showed four additional D_2O exchangeable singlets, attributable to four hydroxy protons in addition to the signals observed for 1. The ^{13}C NMR spectrum (Fig. 4) distortionless enhancement by polarization transfer (DEPT) showed paired singlets corresponding to two methyl, four methylene and six quarternary carbons in accordance with the multiplicity expected based on the parent structure of 1. The most characteristic signals were those that appeared as two sets of paired singlets at δ 106.74 (107.24) and δ 109.25 (110.42) (Fig. 4B). These signals were due to the two acetal carbons, whereas the remaining signals at δ 128.14 (129.49) and δ 154.66 (155.60) were assignable to the olefinic carbons. In GC/MS analysis, the trimethylsilyated derivatives could be separated into two components, and each component showed the $[M - 15]^+$ fragment at *m/z* 431. This fragment also provided further evidence that the oxidation products have two hydroxy groups per molecule.

From the spectral data, we deduced the structures of the oxidation products as methyl (±)-*cis*-2,5-dihydroxy-3-methoxycarbonyl-4-methyl-5-propyl-2,5-dihydro-2-furanpropionate (**3a**) and methyl (±)-*trans*-2,5-dihydroxy-3-methoxycarbonyl-4-methyl-5-propyl-2,5-dihydro-2-furanpropionate (**3b**), respectively (Scheme 1).

The relative ratio of the *cis* and *trans* isomers based on HPLC was 2:1. From molecular modelling, it appears most likely that the major isomer is the *cis*-2,5-dihydroxy-2,5-dihydrofuran (**3a**); the *cis* isomer (**3a**) seems to be favored over the *trans* isomer (**3b**) on account of the enhanced stability of **3a** due to intramolecular hydrogen bonding between the *cis* located dihydroxy groups at C-2 and C-5.

In order to provide further support for the stereochemical relationship between the C-2 and C-5 hydroxy groups, the oxidation reaction was applied to methyl 3-methoxy-

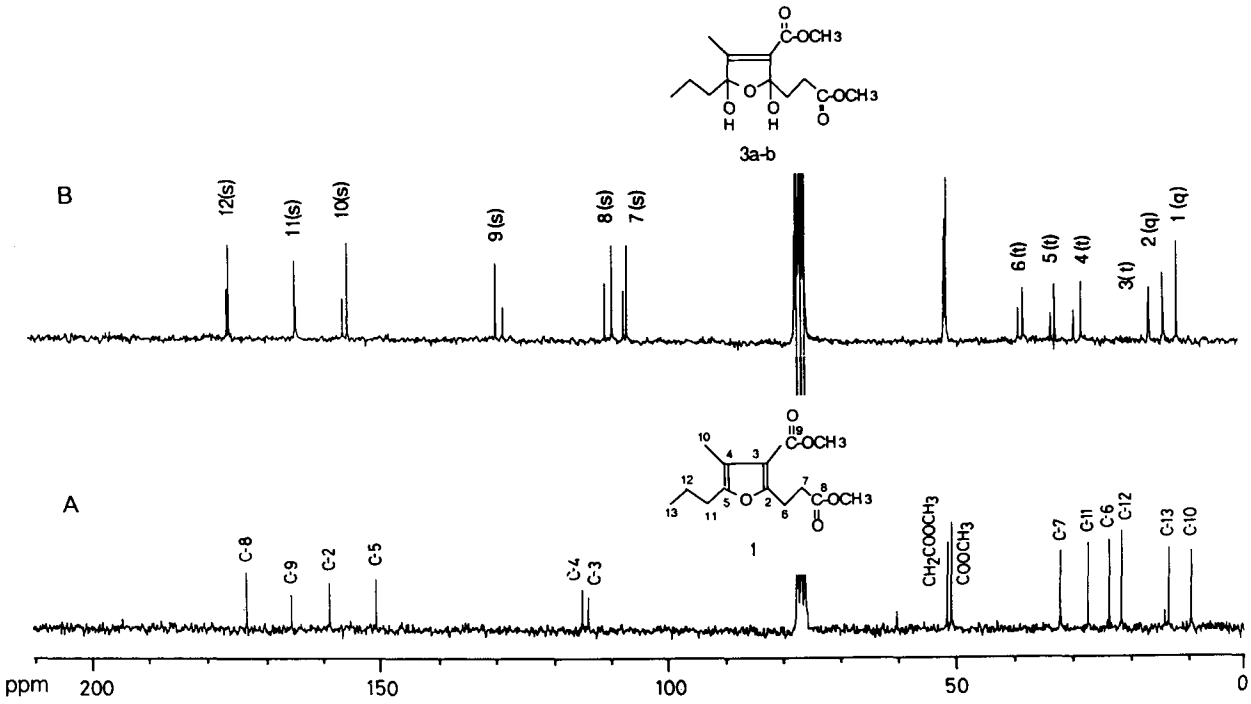


FIG. 4. ^{13}C nuclear magnetic resonance spectra (50.1 MHz, $CDCl_3$) of (A) 1 and (B) oxidation products.

carbonyl-4-methyl-2-furanpropionate (2) which does not bear a 5-alkyl substituent. After a similar workup as for 1, the oxidation products were obtained as an inseparable mixture of two isomers.

The ^1H NMR spectrum (Fig. 5) showed two pairs of doublets (δ 5.56 and δ 5.88) which could be attributed to the 5-hydrogens of the *cis* and *trans* isomers. The 5-hydrogens were coupled to the C-5 geminal hydroxy protons and they changed to singlets when treated with D_2O. The downfield doublet of δ 5.88 was assigned to the *trans* isomer (**4b**), because the 5-hydrogen of **4b** would be deshielded by hydrogen bonding to the C-2 hydroxy proton (Fig. 5). The upfield doublet at δ 5.56 was assigned to the 5-hydrogen of the *cis* isomer (**4a**). The two hydroxy protons at C-5 appeared as doublets at δ 4.09 and δ 4.44. One of hydroxyl protons at C-2 appeared as singlet at δ 4.91, the other C-2 hydroxy proton was absent probably due to overlapping with other signals.

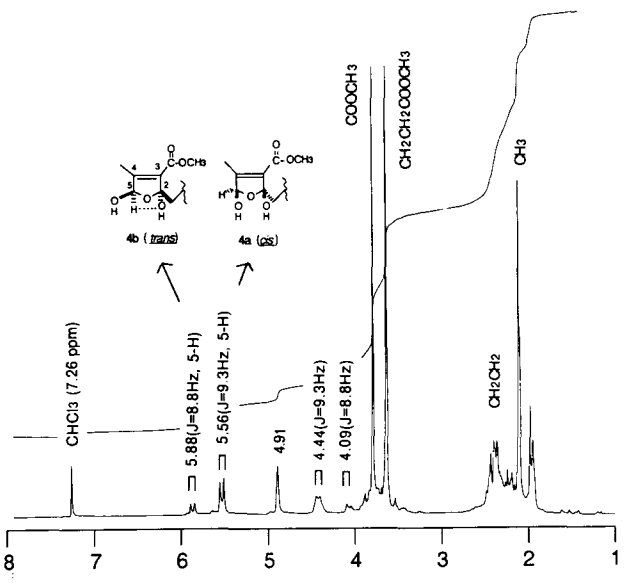


FIG. 5. ^1H nuclear magnetic resonance spectrum (199.5 MHz, $CDCl_3$) of the oxidation products of 2.

The FAB-MS of the mixture showed an $[M - H]^-$ peak at *m/z* 259, consistent with the molecular weight of 2 plus two hydroxy groups. The ^{13}C NMR spectrum was in full agreement with the proposed structures (**4a–b**) (Fig. 6 and Table 1).

Based on these data, the oxidation products were confirmed to be methyl (±)-*cis*-2,5-dihydroxy-3-methoxycarbonyl-4-methyl-2,5-dihydro-2-furanpropionate (**4a**) and methyl (±)-*trans*-2,5-dihydroxy-3-methoxycarbonyl-4-methyl-2,5-dihydro-2-furanpropionate (**4b**), respectively. This structural assignment is consistent with the earlier assignment of the *cis*- and *trans*-2,5-dihydroxy structures **3a** and **3b**.

The relative amounts of **4a** and **4b** based on ^1H NMR data, existed in a ratio of 1:2.11. The major isomer was assumed to be the *cis*-2,5-dihydroxy cmpound (**4a**) because of possible hydrogen bonding between the C-2 and C-5 hydroxy groups located *cis* to each other.

Reaction mechanisms. A reasonable mechanism to account for the formation of the diastereoisomeric 2,5-dihydroxyfuran derivatives is illustrated in Scheme 2. It is interesting to note that the electron withdrawing methoxycarbonyl group at C-3 seems to play an important role in the oxidation reactions. The elimination of hydrogen chloride from the 2-hydroxy-5-chloro intermediate (**7**)

TABLE 1

^{13}C Chemical Shift Assignments for 1 and 2 and Their Products of Oxidation by HOCl

Carbon no.	1	Oxidation products of 1		2	Oxidation products of 2	
		3a	3b		4a	4b
C-2[a]	158.42 (s)[b]	106.74[c] (s)	107.24[c] (s)	161.14 (s)	108.67 (s)	109.08 (s)
C-3	113.60 (s)	129.49 (s)	128.14 (s)	113.40 (s)	129.17 (s)	128.70 (s)
C-4	114.63 (s)	154.66 (s)	155.50 (s)	121.08 (s)	152.64 (s)	153.37 (s)
C-5	150.34 (s)	109.25[c] (s)	110.42[c] (s)	138.10 (d)	101.63 (d)	102.60 (d)
C-6	23.64 (t)	32.99[d] (t)	33.77[d] (t)	23.64 (t)	33.02[d] (t)	33.39[d] (t)
C-7	32.02 (t)	28.43[d] (t)	29.63[d] (t)	31.70 (t)	28.81[d] (t)	29.63[d] (t)
C-8	172.79 (s)	175.07 (s)	175.42 (s)	172.73 (s)	174.45 (s)	175.36 (s)
C-9	165.02 (s)	163.86 (s)	163.68 (s)	164.73 (s)	163.59 (s)	163.59 (s)
C-10	9.63 (q)	11.67 (q)	11.67 (q)	9.89 (q)	12.34 (q)	12.17 (q)
C-11	27.32 (t)	38.36 (t)	39.38 (t)			
C-12	21.57 (t)	16.49 (t)	16.64 (t)			
C-13	13.42 (q)	14.10 (q)	13.98 (q)			
C-8-OCH_3	51.58 (q)	51.88 (q)	51.93 (q)	51.70 (q)	51.85 (q)	51.99 (q)
C-9-OCH_3	50.88 (q)	51.58 (q)	51.58 (q)	51.06 (q)	51.76 (q)	51.76 (q)

[a]Carbon numbers as shown in Scheme 1. [b]Multiplicities were determined by DEPT spectra. [c,d]May be reversed.

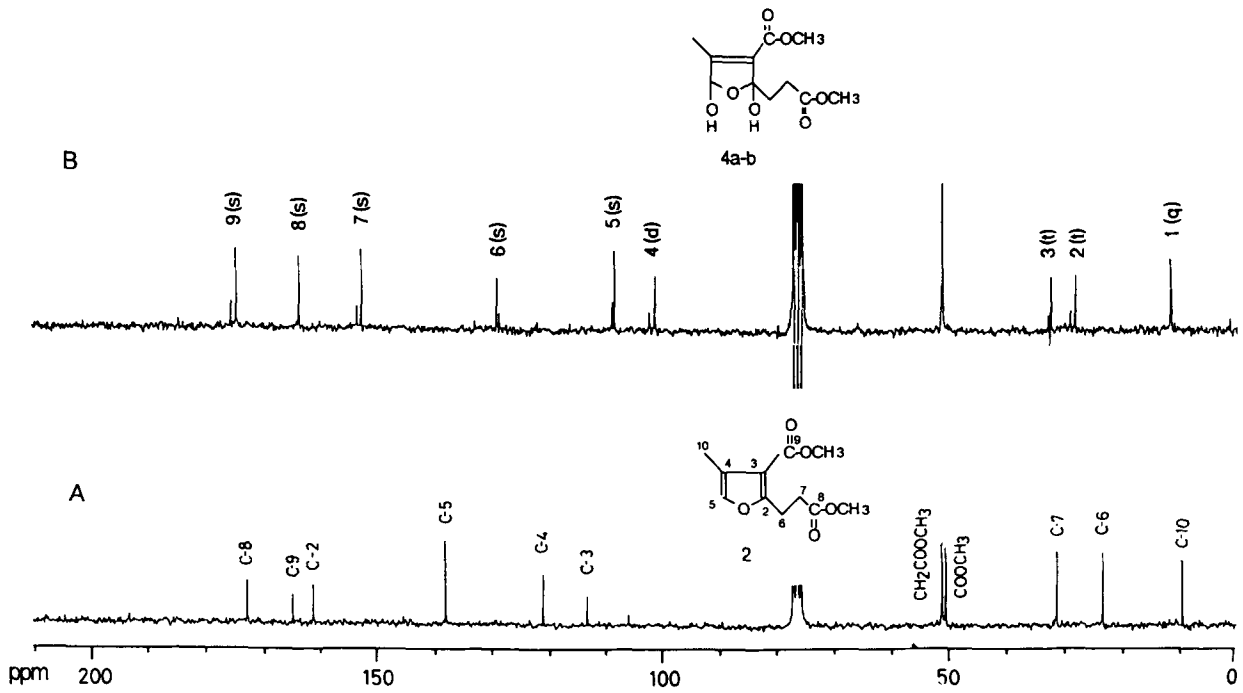
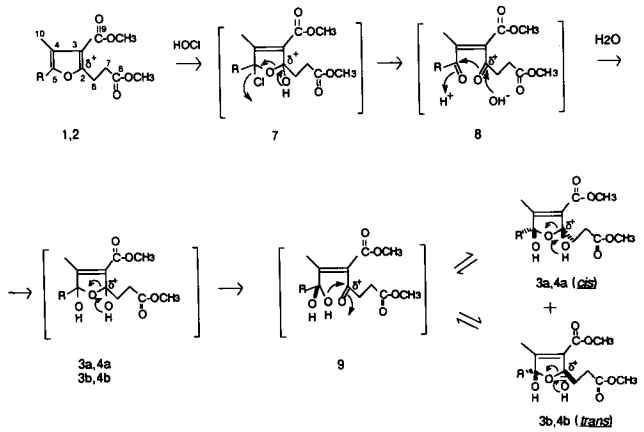


FIG. 6. ^{13}C nuclear magnetic resonance spectra (50.1 MHz, $CDCl_3$) of (A) 2 and (B) oxidation products.

initially formed led to the dioxoene compound (8), consistent with the literature (7-11). The electron deficient carbonyl group of 8 subsequently underwent nucleophilic attack by the hydroxy anion to yield the racemic hemiacetal 3-4, which immediately produced the labile and transient intermediate (9) *via* the subtraction of the hydroxy protons of 3-4 followed by ring-opening. The succeeding reaction of one of the geminal hydroxy groups with the carbonyl group afforded a cyclic hemiacetal accompanied by creation of a new asymmetric center at C-2. This new asymmetric carbon can account for the equilibrium mixture of the *cis* and *trans* stereoisomers (3a-b and 4a-b) due to tautomeric interconversion between the *cis* and *trans* forms. To rationalize this mechanisms, 2,5-dimethylfuran (5) bearing no 3-electron withdrawing substituent was chosen as the model compound and similarly oxidized by HOCl. In this case only *cis*-1,2-diacetylethylene (6) was obtained as the sole product as shown in Scheme 1.

DISCUSSION

We have followed the oxidation of urofuran acid dimethyl esters (1-2) by HOCl; the oxidation was accomplished within 30 s at room temperature. Although we have not



SCHEME 2

been able to isolate the diastereomers, the structures of the oxidation products, **3a–b** and **4a–b** were established by detailed spectroscopic examination.

Initially, we expected that the oxidation would proceed in a fashion similar to that of F-acid to give the corresponding dicarbonyl compounds. However, the oxidation products were found to be very different. The ^{13}C NMR spectra showed no signal that would be attributable to a carbonyl carbon.

The most interesting feature of the reaction mechanism is that the multi-step reaction proceeds under the influence of the electron withdrawing methoxycarbonyl group in 3-position of the furan ring. If the electron withdrawing group is absent, the oxidation will be completed at the initial stage to give the dicarbonyl compound (**8**). It appears that the electron withdrawing effect of the $COOCH_3$ group enhances the nucleophilic attack at the carbonyl group leading to the formation of an equilibrium mixture of diastereoisomers (**3a–b, 4a–b**) after repeated enol-carbonyl nucleophilic additions as shown in **9**.

In summary, this study provides a first example for the effects of an electron withdrawing substituent on the oxidation of urofuran acid dimethyl ester by HOCl. The methodology is simple and accurate and may be useful for the detection of urofuran acid oxidation products.

EXPERIMENTAL PROCEDURES

Materials. Commercial grade solvents and reagents were used without further purification. $CDCl_3$ and silica gel 60 for column chromatography were purchased from E. Merck (Darmstadt, Germany); silica gel K5 for TLC was obtained from Whatman Industrial Ltd. (Maidstone, England).

NaOCl solution. A solution of $Na_2S_2O_3$ ($Na_2S_2O_3 \cdot 5H_2O$, 10 g) in distilled water (400 mL) was calibrated with KIO_3. For this purpose, KIO_3 (102.4 mg, 0.4785 mmol) was dissolved in distilled water (50 mL). KI (2 g) and 50% H_2SO_4 (5 mL) were added to the solution, and the mixture was titrated with $Na_2S_2O_3$ solution. When the color of the solution changed to yellow, 1% soluble starch (1 mL) was added, and the end-point of the titration was determined by disappearance of the blue color. As 1 mol of KIO_3 reacts with 6 mol of $Na_2S_2O_3$, the concentration of $Na_2S_2O_3$ was calculated to be 0.1002 M. Then 1 mL of NaOCl solution was added to 50 mL of distilled water, KI (2 g) and 6 M HCl (2 mL) were added to the solution, and the mixture was titrated with 0.1002 M $Na_2S_2O_3$. As 1 mol NaOCl reacts with 2 mol $Na_2S_2O_3$, the concentration of the NaOCl solution was found to be 1.54 M.

HPLC condition. A model LC-4A chromatograph (Shimadzu, Kyoto, Japan) with a SPD-2AS spectrophotometric detector was used together with an chromatopac C-R2AX integrator. Analyses were done on a TSK gel ODS 80TM (4.6 mm i.d. × 25 cm, particle size 5 μm) column. Acetonitrile/water (55:45, vol/vol) was used as mobile phase at a flow rate of 1.0 mL/min for the analysis of the oxidation products of **1** and **2**; detection wave length was 220 nm and the injection volume was 10 μL.

NMR. ^{13}C NMR and ^1H NMR spectra were recorded on a JNM FX-200 (JEOL, Tokyo, Japan) (50/200 MHz). Chemical shifts are expressed in parts per million (δ) downfield from tetramethylsilane (TMS) unless stated otherwise. DEPT was employed to assign carbon multiplicities (*s*,C; *d*,CH; *t*,CH_2; *q*,CH_3).

Mass spectrometry. Electron impact ionization mass spectra were recorded on a JMS D-300 mass spectrmeter (JEOL) under the following conditions: ionizing energy 70 eV, ionizing current 100 μA, ion source temperature 160°C, and accelerating voltage 3 kV. Chemical ionization mass spectra (CI-MS) using ammonia as a reactant gas were recorded under the same conditions as the EI-MS except for the ionizing energy (200 eV).

Fast atom bombardment mass spectra were recorded on a JMS DX-303 mass spectrometer (JEOL) using the negative ionization mode and a glycerol matrix.

Gas chromatography/mass spectrometry (GC/MS) was performed using a Hewlett-Packard 5710A instrument and a DB1 column (30 m × 0.25 mm i.d.) (Palo Alto, CA) with temperature programming (160°C–250°C at 3°C/min); helium was used as carrier gas at a flow rate of 0.9 mL/min. Oxidation products were trimethylsilylated at 110°C for 60 min with a mixture of TRI-SIL®Z, trimethylchlorosilane and *N,O-bis*-trimethylsilylacetamide in a ratio of 2:1:1 and subjected to GC/MS analysis.

Synthesis of methyl 3-methoxycarbonyl-4-methyl-5-propyl-2-furan propionate (1). CMPF was synthesized according to the method described by Spiteller *et al.* (12) using 3-ketoadipic acid diethyl ester instead of dimethyl ester. A solution of 3-hydroxy-2-hexanone (7.7 g, 66.4 mmol), 3-ketoadipic acid diethyl ester (19.8 g, 91.7 mmol), $ZnCl_2$ (7.0 g), and ethanol (16 mL) were refluxed for 20 h. After pouring into water (50 mL), the reaction mixture was extracted with benzene. The extract was washed successively with 30% $NaHSO_3$, 5% NaOH, and 0.1 M HCl. After dry over anhydrous Na_2SO_4, the extract was evaporated under reduced pressure to give an oil (18.4 g), which was chromatographed on silica gel. Elution with ethyl acetate/hexane (1:9, vol/vol) gave a mixture of ethyl 3-ethoxycarbonyl-4-propyl-5-methyl-2-furanpropionate and ethyl 3-ethoxycarbonyl-4-methyl-5-propyl-2-furanpropionate (9.5 g), which were hydrolyzed by refluxing with a mixture of 10% NaOH (32 mL) and ethanol (324 mL) for 6 h. The mixture acidified to pH 1 with 6 M HCl was extracted with diethyl ether and dried over anhydrous Na_2SO_4. The extract was evaporated to dryness to give a mixture of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid and 3-carboxy-4-propyl-5-methyl-2-furanpropionic acid (4.1 g). Two recrystallizations of the mixture from

chloroform gave 1.56 g (38.0%) of CMPF as a white powder; m.p. 180–182°C. ^{1}H NMR (D_2O, ppm from DSS) δ: 0.87 (3H, *t*, $J = 7.3$ Hz, $CH_2CH_2CH_3$), 1.57 (2H, *m*, $CH_2CH_2CH_3$), 1.98 (3H, *s*, Furan-CH_3), 2.46 (2H, *t*, $J = 7.9$ Hz, CH_2CH_2COOH), 2.52 (2H, *t*, $J = 7.3$ Hz, $CH_2CH_2CH_3$), 3.03 (2H, *t*, $J = 7.9$ Hz, CH_2CH_2COOH). EI-MS *m/z* (relative intensity): 240 (M^+, 10.8), 222(15.7), 211(17.6), 194(39.2), 193(27.5), 181(15.7), 165(100), 149(50.0), 147(11.8), 91(11.8), 77(21.6), 55(31.4).

A solution of CMPF (1.0 g, 4.17 mmol) in a mixture of methanol (12 mL) and benzene (40 mL), was treated with ethereal diazomethane prepared from *N*-methyl-*N*-nitrosourea. After removal of the solvent, the residue was chromatographed on silica gel. Elution with ethyl acetate/hexane (1:4, vol/vol) gave 0.88 g (78.7%) of **1** as a colorless oil. ^{1}H NMR (^{13}C-^{1}H correlation spectroscopy [COSY]) ($CDCl_3$) δ: 0.88 (3H, *t*, $J = 7.3$ Hz, $CH_2CH_2CH_3$), 1.57 (2H, *m*, $CH_2CH_2CH_3$), 2.05 (3H, *s*, Furan-CH_3), 2.45 (2H, *t*, $J = 7.3$ Hz, $CH_2CH_2CH_3$), 2.65 (2H, *t*, $J = 7.7$ Hz, $CH_2CH_2COOCH_3$), 3.24 (2H, *t*, $J = 7.7$ Hz, $CH_2CH_2COOCH_3$), 3.68 (3H, *s*, $CH_2CH_2COOCH_3$), 3.80 (3H, *s*, $COOCH_3$). ^{13}C NMR: see TAble 1. EI-MS *m/z* (relative intensity): 268 (M^+, 29.0), 239(18.8), 237(20.4), 209(19.4), 208(89.2), 207(17.7), 195(52.7), 179(100), 147(12.4), 55(12.9).

Synthesis of methyl 3-methoxycarbonyl-4-methyl-2-furanpropionate (2). 3-Carboxy-4-methyl-2-furan-propionic acid was synthesized by the procedure described by Pfordt *et al.* (13). The crystals appeared as colorless plates; m.p. 188–192°C, ^{1}H NMR (D_2O, ppm from DSS) δ: 2.02 (3H, *d*, $J = 1.2$ Hz, Furan-CH_3), 2.46 (2H, *t*, $J = 7.7$ Hz, CH_2CH_2COOH), 3.05 (2H, *t*, $J = 7.7$ Hz, CH_2CH_2COOH), 7.12 (1H, *q*, $J = 1.2$ Hz, Furan-*H*). EI-MS *m/z* (relative intensity): 198 (M^+, 19.4), 180(11.8), 152(100), 139(32.3), 138(9.7), 135(20.4), 121(7.5), 79(10.8), 77(8.6), 65(9.1).

A solution of 3-carboxy-4-methyl-2-furanpropionic acid (1.1 g, 5.56 mmol) in a mixture of methanol (12 mL) and benzene (40 mL) was treated with ethereal diazomethane prepared from *N*-methyl-*N*-nitrosourea, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give 1.0 g (79.5%) of **2** as a colorless oil. ^{1}H NMR ($CDCl_3$) δ: 2.14 (3H, *d*, $J =$ 1.3 Hz, Furan-CH_3), 2.69 (2H, *t*, 7.7 Hz, $CH_2CH_2COOCH_3$), 3.29 (2H, *t*, 7.7 Hz, $CH_2CH_2COOCH_3$), 3.69 (3H, *s*, $CH_2CH_2COOCH_3$), 3.83 (3H, *s*, $COOCH_3$), 7.06 (1H, *q*, $J = 1.3$ Hz, Furan-*H*). ^{13}C NMR: see Table 1. EI-MS *m/z* (relative intensity): 226 (M^+, 24.6), 195(27.1), 194(25.4), 166(100), 153(45.8), 135(28.8), 134(9.3), 77(6.8), 59(6.8).

Reaction of **1** *with HOCl.* A solution of sodium hypochlorite (154 mM, 14.0 mL) was added with stirring to a mixture of **1** (310 mg, 1.16 mmol), acetone (85 mL) and sodium phosphate buffer (pH 7.40, 170 mL 0.15 M). After stirring for 30 s, the mixture was extracted with diethyl ether (3 × 500 mL). The extracts were dried over Na_2SO_4 and evaporated to dryness. The residual viscous oil was chromatographed on silica gel. Elution with ethyl acetate/hexane (7:3, vol/vol) gave three fractions: Fraction 5 (26.3 mg, 8.4%, R_f 0.97), Fractions 6–8 (172.0 mg, 54.8%, R_f 0.97, 0.91, 0.57) and Fractions 9–14 (115.5 mg, 36.8%, R_f 0.79 and 0.57). Effluents were gathered in a fraction collector in 9-mL volumes. One μL of each fraction was applied to a TLC plate which was developed with ethyl acetate/hexane (7:3, vol/vol). Fractions were detected by treatment with 50% H_2SO_4 and heating at 150°C for 3 min. Fractions 9–14: ^{1}H NMR ($CDCl_3$) δ: 0.90 (3H, *t*, $J = 7.0$ Hz, $CH_2CH_2CH_3$), 1.2–2.0 (4H, *m*, $CH_2CH_2CH_3$ and $CH_2CH_2CH_3$), 2.07 (3H, *s*, Furan-CH_3), 2.2–2.6 (4H, *m*, $CH_2CH_2COOCH_3$ and $CH_2CH_2COOCH_3$), 3.39 (1H, *s*, OH of **3b**, D_2O exchangeable), 3.63 (3H, *s*, $CH_2CH_2COOCH_3$ of **3b**), 3.67 (3H, *s*, $CH_2CH_2COOCH_3$ of **3a**), 3.80 (3H, *s*, $COOCH_3$), 3.95 (1H, *s*, OH of **3b**, D_2O exchangeable), 4.10 (1H, *s*, OH of **3a**, D_2O exchangeable), 5.18 (1H, *s*, OH of **3a**, D_2O exchangeable). ^{13}C NMR data are summarized in Table 1. Negative FAB-MS *m/z*: 301 ($[M - H]^-$), 284 ($[M - H]^- - OH$), 283 ($[M - H] - H_2O$), 269 ($[M - H]^- - CH_3OH$); CI-MS (ammonia) *m/z*: 302; EI-MS *m/z* (relative intensity): 284 ($M^+ - H_2O$, 5.0), 266(33.7), 253(19.1), 241(56.3), 237(12.6), 210(14.4), 209(29.4), 207(20.7), 206(28.9), 193(23.0), 181(100), 165(31.6), 149(19.0), 115(32.0), 71(15.2), 67(20.3), 59(17.5), 55(36.7). *anal.* Calcd for $C_{14}H_{22}O_7$: C, 55.62; H, 7.34. Found: C, 54.83; H, 7.29, GC/MS analysis of the trimethylsilylated derivatives showed two components in a ratio of 1:1.28 with retention times of 13.9 min and 15.3 min, respectively. Component with retention time 13.9 min *(trans):* EI-MS *m/z* (relative intensity): 431 (M − 15, 7.9), 403(63.4), 359(53.0), 313(27.3), 281(15.7), 269(18.9), 267(19.2), 235(14.4), 207(11.4), 181(23.8), 165(36.2), 147(12.5), 89(14.4), 75(46.6), 73(100); CI-MS (ammonia) *m/z*: 357 ($MH^+ - (CH_3)_3SiOH$), 284 ($MNH_4^+ - (CH_3)_3SiOH - (CH_3)_3SiOH$), 267 ($MH^+ - (CH_3)_3SiOH - (CH_3)_3SiOH$). Component with retention time 15.3 min *(cis):* EI-MS *m/z* (relative intensity): 431 ($M^+ - 15$, 5.0), 403(56.5), 359(46.0), 313(23.7), 281(14.2), 269(16.3), 267(30.7), 235(19.0), 207(9.7), 181(22.5), 165(34.8), 147(19.1), 89(13.7), 75(38.7), 73(100); CI-MS *m/z* 357 ($MH^+ - (CH_3)_3SiOH$), 284 ($MNH_4^+ - (CH_3)_3SiOH - (CH_3)_3SiOH$), 267 ($MH^+ - (CH_3)_3SiOH - (CH_3)_3SiOH$).

Reaction of **2** *with HOCl.* A solution of sodium hypochlorite (1.54 M, 700 μL) was added with stirring to a mixture of **2** (230 mg, 1.02 mmol), acetone (16 mL), and sodium phosphate buffer (pH 7.40, 46 mL of 0.15 M). After stirring for 30 s, the reaction mixture was extracted with diethyl ether (150 mL × 3), and the extracts were dried over Na_2SO_4 and evaporated under reduced pressure at 10°C to give an oil, which was chromatographed on silica gel. Elution with ethyl acetate/hexane (7:3, vol/vol) gave a colorless viscous oil (97 mg). ^{1}H NMR ($CDCl_3$) δ: 2.13 (3H, *s*, Furan-CH_3), 2.2–2.5 (4H, *m*, CH_2CH_2 and CH_2CH_2), 3.66 (3H, *s*, $CH_2CH_2COOCH_3$), 3.81 (3H, *s*, $COOCH_3$), 4.09 (1H, *d*, $J = 8.8$ Hz, 5-OH of **4b**), 4.44 (1H, *d*, $J = 9.3$ Hz, 5-OH of **4a**), 4.91 (1H, *s*, 2-OH of **4a** or **4b**), 5.56 (1H, *d*, $J = 9.3$ Hz, 5-H of **4a**), 5.88 (1H, *d*, 8.8 Hz, 5-H of **4b**). ^{13}C NMR: see Table 1. Negative FAB-MS *m/z*: 259 ($[M - H]^-$), 242 ($[M - H]^- - OH$), 241 ($[M - H]^- - H_2O$), 227 ($[M - H]^- - CH_3OH$); CI-MS (ammonia) *m/z:* 260; EI-MS *m/z* (relative intensity): 242 ($M^+ - H_2O$, 25.5), 211 (38.6), 210(100), 183(33.7), 182(45.2), 173(21.7), 155(29.2), 151(20.0), 150(52.1), 127(60.2), 123(53.2), 122(21.1), 115(65.0), 95(22.1), 67(29.6), 59(46.1), 55(63.3). GC/MS analysis of the trimethylsilylated derivatives showed two components in a ratio of 1:2.20 with retention times of 10.2 min and 11.0 min, respectively. Component with retention time 10.2 min *(trans):* EI-MS *m/z* (relative intensity): 403 ($M^+ - H$, 1.0), 389 ($M^+ - 15$, 5.4), 317(91.7), 315(6.3), 299(7.3), 241(16.7), 193(6.3), 185(33.3), 169(12.5), 147(15.6), 127(12.5), 123(13.5), 89(16.7), 75(27.1), 73(100); CI-MS (ammonia) *m/z:* 422 (MNH_4^+), 322 ($MNH_4^+ - (CH_3)_3SiOH$), 315

$(MH^+ - (CH_3)_3SiOH)$, 260 $(242 + NH_4)$, 242 $(NH_4^+ - (CH_3)_3SiOH - (CH_3)_3SiOH)$. Component with retention time 11.0 min *(cis):* EI-MS *m/z* (relative intensity): 403 $(M^+ - H, 1.0)$, 389(6.0), 317(82.8), 315(10.9), 299(17.2), 241(11.2), 193(10.3), 185(32.8), 169(16.4), 147(30.2), 127(12.9), 123(20.7), 89(20.7), 75(30.2), 73(100); CI-MS (ammonia) *m/z*: 422 (MNH_4^+), 322 $(MNH_4^+ - (CH_3)_3SiOH)$, 315 $(MH^+ - (CH_3)_3SiOH)$, 260 $(242 + NH_4)$, 242 $(NH_4^+ - (CH_3)_3SiOH - (CH_3)_3SiOH)$.

Reaction of 5 *with HOCl.* A solution of sodium hypochlorite (0.154 M, 40.6 mL) was added with stirring to a mixture of 5 (610 mg, 6.35 mmol), acetone (255 mL), and sodium phosphate buffer (pH 7.40, 510 mL of 0.15 M). After 30 s, the mixture was extracted with diethyl ether (1 L × 3). The extracts were dried over Na_2SO_4 and concentrated under reduced pressure below 10°C to give a residue which was chromatographed on silica gel. Elution with ethyl acetate/hexane (4:6, vol/vol) gave 287 mg (40.3%) of *cis*-1,2-diacetylethylene (**6**) as a liquid (287 mg). ^1H NMR ($CDCl_3$) δ: 2.26 (6H, *s*, $2 \times CH_3$), 6.28 (2H, *s*, CH=CH). ^{13}C NMR ($CDCl_3$) δ: 29.57 (CH_3), 135.50 (CH=CH), 200.27 (C=O). EI-MS *m/z* (relative intensity): 112 (M^+, 20.5), 97 (54.0), 69 (14.2), 43 (100).

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Synthesis of Phenyl Substituted C_{18} Furanoid Fatty Esters

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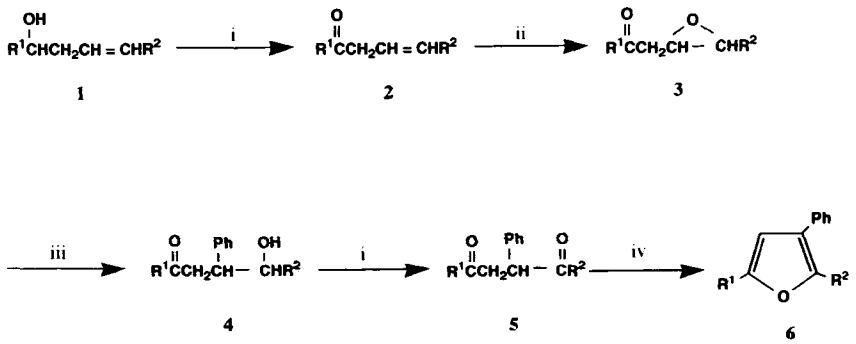
Methyl 9,12-epoxy-10-phenyl-9,11-octadecadienoate was prepared by acid catalyzed cyclization of methyl 9,12-dioxo-10-phenyloctadecanoate, which was derived from the oxidation of methyl 9-hydroxy-12-oxo-10-phenyloctadecanoate. The latter was exclusively obtained from methyl *cis*-9,10-epoxy-12-oxooctadecanoate with phenyllithium in the presence of copper (I) bromide. A mixture of positional isomers, methyl 9,12-epoxy-10(11)-phenyl-9,11-octadecadienoates, was also prepared by another route. The spectroscopic properties of the various intermediates and products were studied. The positional isomers of the phenyl substituted furanoid fatty esters were characterized by ^{13}C nuclear magnetic resonance spectrometry.
Lipids 28, 43–46 (1993).

Long-chain fatty acids containing a furan nucleus in the alkyl chain have been found in the lipid extracts of fish (1–5), plants (6–9), soft corals (10), crayfish (11), amphibians and reptiles (12) and in human and bovine blood plasma (13,14). Biosynthetic experiments by Sand *et al.* (15) have shown that furanoid fatty acids in fish are not produced from acetate by *de novo* synthesis, while Gorst-Allman *et al.* (16) have ruled out 10,13-nonadecadienoic acid as a possible precursor of furanoid fatty acids in animals.

In natural furanoid fatty acids, the only substituent found at the 3- and/or 4-position of the furan ring is a methyl group. We have reported the synthesis of the mono- and dimethyl substituted C_{18} furanoid fatty acid derivatives (17,18) and have recently extended this work to show the possible conversion of a malonic acid group to a methyl substituent (19). In the present paper, we describe the synthesis of a C_{18} furanoid fatty ester derivative containing a phenyl group in the 3-position of the furan ring (Scheme 1) and another procedure for the preparation of a mixture of positional isomers of C_{18} furanoid fatty esters containing a phenyl group at either the 3- or 4- position of the furan ring (Scheme 2).

MATERIALS AND METHODS

Thin-layer chromatography (TLC) was carried out on silica gel coated microscope slides (*ca.* 0.1 mm layers). Preparative TLC separations were done on glass plates (20 × 20 cm) coated with silica gel (0.7-mm layers). Column chromatographic purifications were done on silica gel, and mixtures of light petroleum (b.p. 60–80°C)/diethyl ether were used as eluent. Elemental analyses were carried out by Butterworth Laboratories Ltd. (Teddington, Middlesex, England, U.K.). Infrared spectra were obtained with a Perkin-Elmer 577 (Perkin-Elmer, Norwalk, CT) spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded with a JEOL FX-90Q (90 MHz) (JEOL Ltd., Tokyo, Japan) Fourier transform spectrometer at 89.56 MHz for protons and at 22.5 MHz for carbon. Chemical shifts are expressed as ppm relative to tetramethylsilane used as an internal standard. Methyl ricinoleate (methyl 12-hydroxy-*cis*-9-octadecenoate) (1) was obtained from castor oil. Methyl 12-oxo-9-*cis*-octadecenoate (2), methyl *cis*-9,10-epoxy-12-oxooctadecanoate (3) and methyl 9,12-epoxy-9,11-octadecadienoate (7) were prepared by methods described elsewhere (18). Benzohydroxamic acid was prepared according to the procedure described by Hauser and Renfrow (20). Phenyllithium in



$R^1 = CH_3(CH_2)_5$

$R^2 = (CH_2)_7COOCH_3$

Reagents:
i, H_2CrO_4, Et_2O
ii, *m*-chloroperoxybenzoic acid, CH_2Cl_2
iii, Ph_2CuLi, Et_2O
iv, BF_3-Et_2O, $CHCl_3$

SCHEME 1

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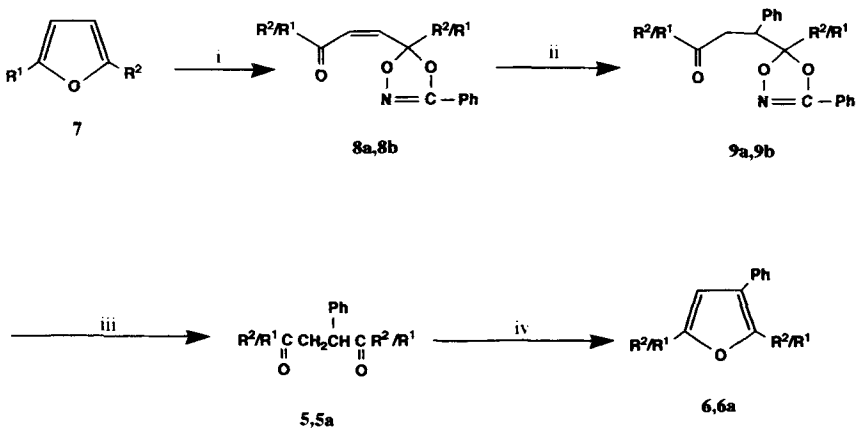
Abbreviations: IR, infrared spectroscopy; NMR, nuclear magnetic resonance; PE, mixture of light petroleum and diethyl ether [PE30 denotes a mixture of light petroleum/diethyl ether (70:30, vol/vol) where the numeral stands for the percentage of diethyl ether in the solvent mixture]; R_f, retardation factor; TLC, thin-layer chromatography.



$R^1 = CH_3(CH_2)_5$

$R^2 = (CH_2)_7COOCH_3$

Reagents:
i, benzohydroxamic acid, Pb (IV) oxide
ii, Ph_2CuLi, Et_2O
iii, EtOH, H_2O
iv, BF_3-OEt_2, $CHCl_3$

SCHEME 2

cyclohexane/ether was purchased from Aldrich Chemical Co. (Milwaukee, WI). All solvents were distilled and dried before use.

Preparation of methyl 9-hydroxy-12-oxo-10-phenyloctadecanoate (4). Phenyllithium in cyclohexane/ether (1.8 M, 6.4 mL) was added dropwise to a suspension of copper(I) bromide (0.83 g, 5.8 mmol) in diethyl ether (20 mL) at 0°C under nitrogen and stirred for 30 min. Methyl *cis*-9,10-epoxy-12-oxooctadecanoate (3, 1.5 g, 4.6 mmol) in anhydrous diethyl ether (30 mL) was added, and the reaction mixture stirred for a further 2 h at 0°C. Saturated aqueous ammonium chloride solution (50 mL) was added, and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic extract was washed with brine (2 × 20 mL) and was dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was chromatographed on a silica column (100 g) using a mixture of light petroleum/diethyl ether (3:2, vol/vol) as eluant to give methyl 9-hydroxy-12-oxo-10-phenyloctadecanoate (4, 0.96 g, 52%) as an oil. Retardation factor (R_f) 0.3 (PE30); infrared (IR) (cm^{-1}) (film) 3400(*s*, O-H stretching), 1740(*s*, C=O stretching), 1580(*m*), 1450(*m*); ^1H NMR ($CDCl_3$, δ): 0.87 (*t*, 3H, CH_3), 1.2–1.8 (*m*, 18H, CH_2), 2.30 (*t*, $J = 7.5$ Hz, 2H, 2-*H*), 2.4–2.6 (*m*, 7H), 3.66 (*s*, 3H, $COOCH_3$), 4.05 (*q*, 1H, $J = 7.0$ Hz, *CH*OH), 6.7–6.9 (*m*, 3H, arom.) and 7.0–7.3 (*m*, 2H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.0 (C-18), 22.5 (C-17), 23.6 (C-14), 24.9 (C-3), 25.6 (C-7), 29.0, 29.1, 29.3, 31.6 (C-16), 34.1 (C-2), 34.4 (C-8), 43.6 (C-13), 45.2 (C-11), 46.2 (C-10), 51.4 ($COOCH_3$), 60.3 (C-9), 128.4 (C-4′ arom.), 128.7 (C-2′ arom.), 129.0 (C-3′ arom.), 129.4 (C-1′ arom.), 174.5 (C-1) and 210.9 (C-12).

Preparation of methyl 9,12-dioxo-10-phenyloctadecanoate (5). Compound 4 (0.18 g, 0.44 mmol) was dissolved in diethyl ether (80 mL) and chromic acid (2.0 mL, prepared from 20 g of sodium dichromate, 28 g sulfuric acid and 65 mL water) was added over a period of 5 min at room temperature. The reaction mixture was stirred for a further 30 min. The ethereal solution was washed with water (20 mL) and then with sodium hydrogencarbonate (10%, 20 mL). The organic mixture was dried over anhydrous sodium sulfate. Silica column chromatographic purification gave methyl 9,12-dioxo-10-phenyloctadecanoate (5, 0.17 g, 95%) as an oil. R_f 0.5 (PE30); ^1H NMR ($CDCl_3$, δ): 0.87 (*t*, 3H, CH_3), 1.2–1.8 (*m*, 18 H, CH_2), 2.2–2.6 (*m*, 6H, CH_2), 3.39 (*d*, $J = 10.3$ Hz, 1H, CH_2CHPh), 3.43 (*d*, $J = 10.3$ Hz, 1H, CH_2CHPh), 3.66 (*s*, $COOCH_3$), 4.20, 4.23 (*d*, $J = 3.9$ Hz, 1H, *CH*Ph) and 7.1–7.5 (*m*, 5H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.0 (C-18), 22.4 (C-17), 23.6 (C-14), 23.8 (C-7), 24.9 (C-3), 28.6, 28.7, 28.9, 31.5 (C-16), 34.3 (C-2), 41.6, 42.9 (C-8/C-13), 45.8 (C-11), 51.4 ($COOCH_3$), 53.3 (C-10), 127.4 (C-4′ arom.), 128.3 (C-2′ arom.), 129.0 (C-3′ arom.), 138.1 (C-1′ arom.), 174.4 (C-1), 209.8 and 210.0 (C-9/C-12).

Preparation of methyl 9,12-epoxy-10-phenyl-9,11-octadecadienoate (6). A mixture of compound 5 (0.15 g, 0.37 mmol), boron trifluoride etherate (0.2 mL) and chloroform (10 mL) was stirred at room temperature for 3 h. Water (20 mL) was added and the chloroform layer was isolated. The organic layer was then dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure, and the residue was separated by preparative TLC using a mixture of light petroleum/diethyl ether, (95:5, vol/vol) as developing solvent to give methyl 9,12-epoxy-10-phenyl-9,11-octadecadienoate (6, 0.13 g, 93%). Elemental analysis: Calc. for $C_{25}H_{36}O_3$ (%), C, 78.08; H, 9.44; O, 12.48; found, C, 78.18; H, 9.41; O, 12.50. R_f 0.8 (PE20); IR (cm^{-1}) (film): 3100 (*w*, C-H furan and arom.), 1740 (*s*, C=O stretching), 1560, 1442, 1010 (*m*, C-O-C furan), 790 and 730; ^1H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.2–1.4 (*m*, 12H, CH_2), 1.4–1.8 (*m*, 6H, CH_2), 2.29 (*t*, 2H, 2-*H*), 2.60 (*t*, $J = 7.0$ Hz, 2H, 13-*H*), 2.73 (*t*, $J = 7.0$ Hz, 2H, 8-*H*), 3.65 (*s*, 3H, $COOCH_3$), 6.07 (*s*, 1H, furan *CH*) and 7.33 (*s*, 5H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.1 (C-18), 22.6 (C-17), 24.9 (C-3), 27.0 (C-8), 28.0 (C-13), 28.1, 28.6, 29.0, 31.6 (C-16), 34.1 (C-2), 51.4 ($COOCH_3$), 106.2 (C-11), 121.2 (C-10), 126.0 (C-4′ arom.), 127.6 (C-2′ arom.), 128.4 (C-3′ arom.), 134.8 (C-1′ arom.), 149.8 (C-9), 154.3 (C-12) and 174.2 (C-1).

Preparation of methyl dioxazolyl C_{18}-enone esters (8a,8b). A mixture of methyl 9,12-epoxy-9,11-octadecadienoate (7, 14 g, 45.5 mmol), benzohydroxamic acid (9 g,

65 mmol), lead(IV) oxide (60 g, 0.25 mol) and ethyl acetate (150 mL) was stirred at room temperature for 24 h. The reaction mixture was filtered and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica column (200 g) using a mixture of light petroleum/diethyl ether (7:3, vol/vol) as eluant to give compounds **8a,8b** (14.7 g, 73%) as an oil. R_f 0.35 (PE40); IR (cm^{-1}) (film): 1735 (*s*, C=O stretching, ester), 1690 (*s*, C=O stretching, oxo), 1620 (m), 1450, 1360, 760, 690; ^1H NMR ($CDCl_3$, δ): 0.87 (*t*, 3H, CH_3), 1.2-1.4 (*m*, 12H, CH_2), 1.6-1.8 (*m*, 6H, CH_2), 2.20 (*t*, 2H, $J = 7.5$ Hz, $CH_2COOCNPh$), 2.30 (*t*, 2H, 2-*H*), 2.51 (*t*, $J = 7.5$ Hz, 2H, $COCH_2$), 3.66 (*s*, 3H, $COOCH_3$), 5.84, 6.18 (2*d*, $J = 12.8$ Hz, 2H, CH=CH), 7.3-7.5 (*m*, 3H, arom.) and 7.6-7.8 (*m*, 2H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.0 (C-18), 22.5 (C-17), 23.4 (C-7 or C-14), 24.9 (C-3), 28.9, 29.0, 29.1, 29.3, 31.7 (C-16), 34.0 (C-2), 37.7, 37.8, ($CH_2COOCNPh$), 43.3, 43.5 ($COCH_2$), 51.2 ($COOCH_3$), 115.4 (COOCNPh), 123.4 (C-4′ arom.), 126.8 (C-2′ arom.), 128.7 (C-3′ arom.), 131.4 (C-1′ arom.), 132.4, 132.7 (C-10 or C-11), 158.1 (C=N), 173.8 (C-1) and 203.0 (C=O, oxo).

Preparation of phenyl substituted dioxazolyl C_{18}-keto esters (**9a,9b**). A mixture of freshly prepared copper(I) bromide (0.85 g, 5.9 mmol), phenyllithium (6.6 mL of a 1.8 *M solution) and anhydrous diethyl ether (150 mL)* was stirred at 0°C under nitrogen for 15 min. The mixture of compounds **8a,8b** (2.0 g, 4.5 mmol) was added and the temperature maintained at 0-5°C for 1 h. The reaction mixture was stirred for 10 h at room temperature and was then poured into cold aqueous ammonium chloride (2.0 M, 150 mL). The product was isolated by extraction with diethyl ether (2 × 100 mL) and was dried over anhydrous sodium sulfate. The solvent was evaporated, and silica gel column chromatography of the residue yielded a mixture of compounds **9a,9b** (1.8 g, 80%) as an oil. R_f 0.3 (PE40); IR (cm^{-1}) (film): 1734 (*s*), 1690 (*s*), 1615, 1570, 1440, 790, 720; ^1H NMR ($CDCl_3$, δ): 0.81 (*t*, 3H, CH_3), 1.2-1.8 (*m*, 20H, CH_2), 2.1-2.4 (*m*, 4H, CH_2), 2.90 (*m*, 2H, CH_2CHPh), 3.65 (*s*, 3H, $COOCH_3$), 3.78 (*dd*, $J = 4.0$, 9.0 Hz, 1H, CHPh) and 7.1-7.8 (*m*, 10H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 13.9 (C-18), 22.4 (C-17), 23.5 (CH_2CH_2CO), 24.8 (C-3), 28.7, 28.8, 28.9, 29.1, 29.2, 31.5 (C-16), 34.1 (C-2), 36.4 (C-8 or C-13), 42.8 (CH_2CHPh), 43.4 (CH_2CO), 47.8 (CHPh), 51.4 ($COOCH_3$), 119.8 (COOCNPh), 123.1 (arom.), 126.7 (arom.), 127.3 (arom.), 128.4 (arom.), 128.7 (arom.), 129.1 (arom.), 131.4 (arom.), 138.9 (arom.), 158.6 (C=N) and 174.1 (C-1).

Preparation of methyl 9,12-dioxo-10(11)-phenyloctadecanoates (**5,5a**). A mixture of compounds **9a,9b** (1.5 g, 2.8 mmol and aqueous ethanol (50%, 60 mL) was refluxed for 20 h. Water (50 mL) was added, and the reaction mixture extracted with diethyl ether (2 × 50 mL). The ethereal extract was washed with water (20 mL) and was dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was chromatographed on a silica gel column using a mixture of petroleum ether/diethyl ether (4:1, vol/vol) as eluent to give a mixture of methyl 9,12-dioxo-10(11)-phenyloctadecanoates (**5,5a**) (0.97 g, 86%) as an oil. R_f 0.30 (PE40); IR (cm^{-1}) (film): 1740 (*s*, C=O stretching, ester), 1703 (*s*, C=O stretching, oxo), 1565, 1446, 790, 730. ^1H NMR ($CDCl_3$, δ): 0.87 (*t*, 3H, CH_3), 1.2-1.8 (*m*, 18H, CH_2), 2.2-2.6 (*m*, 6H, CH_2), 3.39 (*d*, $J = 10.3$ Hz, 1H, CH_2CHPh), 3.43 (*d*, $J = 10.3$ Hz, 1H, CH_2CHPh), 3.66 (*s*, 3H, $COOCH_3$), 4.20, 4.23 (*d*, $J = 3.9$ Hz, 1H, CHPh) and 7.1-7.5 (*m*, 5H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.0 (C-18), 22.4 (C-17), 23.6, 23.8 (CH_2CO), 24.9 (C-3), 28.6, 28.7, 28.9, 31.5 (C-16), 34.3 (C-2), 41.6, 42.9 (CH_2CO), 45.8 ($COCH_2CHPh$), 51.4 ($COOCH_3$), 53.3 (CHPh), 127.4 (C-4′ arom.), 128.3 (C-2′ arom.), 129.0 (C-3′ arom.), 138.1 (C-1′ arom.), 174.4 (C-1), 209.8 and 210.0 (C=O, oxo).

Preparation of methyl 9,12-epoxy-10(11)-phenyl-9,11-octadecadienoates (**6,6a**). A mixture of methyl 9,12-dioxo-10(11)-phenyloctadecanoates (**5,5a**) (1.0 g, 2.6 mmol), boron trifluoride etherate (0.5 mL) and chloroform (20 mL) was stirred at room temperature for 3 h. Aqueous sodium carbonate (10%, 10 mL) was added, and the organic mixture was extracted with diethyl ether (2 × 20 mL). The solvent was evaporated and silica gel column chromatography of the residue gave compounds (**6,6a**) as an oil (0.96 g, 96%). R_f 0.8 (PE30); IR (cm^{-1}) (film): 1740 (*s*, C=O stretching), 1560 (*m*), 1142, 1010 (*w*), 790, 730; ^1H NMR ($CDCl_3$, δ): 0.88 (*t*, 3H, CH_3), 1.2-1.4 (*m*, 12H, CH_2), 1.4-1.8 (*m*, 6H, CH_2), 2.29 (*t*, 2H, 2-*H*), 2.60 (*t*, $J = 7.5$ Hz, 2H), 2.73 (*t*, $J = 6.8$ Hz, 2H), 3.65 (*s*, 3H, $COOCH_3$), 6.07 (*s*, 1H, furan CH) and 7.2-7.4 (*m*, 5H, arom.); ^{13}C NMR ($CDCl_3$, ppm): 14.1 (C-18), 22.6 (C-17), 24.9 (C-3), 26.9, 27.0 (α-CH_2 to furan), 28.0, 28.6, 29.0, 31.6 (C-17), 34.1 (C-2), 51.4 ($COOCH_3$), 106.0, 106.2 (CH furan), 121.2 (C-Ph), 126.0 (C-1′ arom.), 127.6 (C-2′ arom.), 128.4 (C-3′ arom.), 134.8 (C-4′ arom.), 149.8, 150.0, 154.1, 154.3 (C-2′ furan) and 174.1 (C-1).

RESULTS AND DISCUSSION

Following our earlier success in the regiospecific methylation of methyl *cis*-9,10-epoxy-12-oxooctadecanoate (**3**) (18), the latter compound was reacted with lithiumdiphenyl cuprate [generated *in situ* from phenyllithium and copper(I) bromide] to yield methyl 9-hydroxy-12-oxo-10-phenyloctadecanoate (**4**, 52%), exclusively. Two-phase oxidation of compound **4** gave methyl 9,12-dioxo-10-phenyloctadecanoate (**5**, 95%). Compound **5** was cyclized in the presence of boron trifluoride etherate to methyl 9,12-epoxy-10-phenyl-9,11-octadecadienoate (**6**, 93%).

The infrared spectrum of compound **6** showed a weak absorption band at 3100 cm^{-1}, while the ester carbonyl function absorbed at 1740 cm^{-1}. The presence of the phenyl system in methyl 9,12-epoxy-10-phenyl-9,11-octadecadienoate (**6**) was apparent from the appearance of the signal at δ 7.33 (*s*, 5H) arising from the shift of the aromatic protons in the ^1H NMR spectrum. The furan proton (11-*H*) appeared at δ 6.07 (*s*, 1H). The effect of the phenyl group at the C-10 position caused the protons of the methylene groups adjacent to the furan nucleus to be chemically nonequivalent. The shifts of the 8-*H* and 13-*H* protons were found at δ 2.73 and 2.60, respectively.

In the ^{13}C NMR spectral analysis of compound **6**, the monosubstituted phenyl system was confirmed by the appearance of four signals in the aromatic region, *viz.* 126.0 (C-4′ arom.), 127.6 (C-2′ arom.), 128.4 (C-3′ arom.) and 134.8 (C-1′ arom.). The effect of the phenyl ring on the chemical shifts of the furan carbon atoms was very significant. The carbon atoms of the furan nucleus were shifted to 149.8 (C-9), 121.2 (C-10) and 154.3 (C-12), while the methine carbon of the furan appeared at 106.2 (C-11) ppm. The shifts of the methylene carbon atoms (C-8, C-13) adjacent to the furan nucleus were also affected by the phenyl substituent in the furan nucleus. The chemical shifts for the C-8 and

C-13 carbon atoms were found at 27.0 and 28.0 ppm, respectively.

In the preparation of a mixture of positional isomers of furanoid fatty ester derivatives containing a phenyl group at the 3- or 4- position of the furan nucleus (**6,6a**), methyl 9,12-epoxy-9,11-octadecadienoate (**7**) was stirred with benzohydroxamic acid in the presence of lead(IV) oxide. Ring opening of the furan ring resulted in the formation of a mixture of conjugated dioxazolyl C_{18}-enone intermediates (**8a,8b**, 73%) (21). This reaction was nonregiospecific, and compounds **8a,8b** contain an oxo group at either the C-9 or C-12 position of the alkyl chain. Treatment of the mixture of compounds **8a,8b** with phenyllithium in the presence of copper(I) bromide furnished the corresponding 1,4-Michael addition products (**9a,9b**, 80%). The dioxazolyl function was subsequently removed by refluxing with aqueous ethanol to yield methyl 9,12-dioxo-10(11)-phenyloctadecanoates (**5,5a**, 86%). The latter mixture of compounds was treated with boron trifluoride etherate in chloroform to furnish a mixture of positional isomers of phenyl furanoid fatty esters, methyl 9,12-epoxy-10(11)-phenyl-9,11-octadecadienoates (**6,6a**, 96%).

In the analysis of the mixture of compounds **6,6a**, the infrared and ^{1}H NMR spectra were identical to those recorded for the single isomer **6**. However, the presence of the two positional isomers (**6,6a**) in the mixture was apparent from the ^{13}C NMR spectral analysis. The chemical shifts of the furan carbon nuclei were resolved into seven signals (106.0, 106.2, 121.2, 149.8, 150.0, 154.1 and 154.3 ppm). As the shift values of the furan carbon nuclei (106.2, 121.2, 149.8 and 154.3 ppm) for compound **6** were already established, it was therefore possible to assign unequivocally the shifts of the carbon nuclei of the furan nucleus of compound **6a**. Thus, the chemical shifts of the furan carbon atoms of compound **6a** (methyl 9,12-epoxy-11-phenyl-9,11-octadecadienoate) appeared at 154.1, 106.0, 121.2 and 150.0 ppm for C-9, C-10, C-11 and C-12, respectively. ^{13}C NMR spectrometry thus permitted the full characterization of C_{18} furanoid fatty ester derivatives containing a phenyl substituent at the 3- or 4-position of the furan nucleus.

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Preparation of Methyl 5,11,14,17-Eicosatetraenoate-8,8,9,9-d_4

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For studies of incorporation, elongation and desaturation of fats in humans and animals, methyl 5,11,14,17-eicosatetraenoate-8,8,9,9-d_4 was prepared by Wittig coupling, in the presence of sodium *bis*(trimethylsilyl) amide, of 6,9,12-pentadecatrienal-3,3,4,4-d_4 and 4-carboxybutyltriphenylphosphonium bromide. The all-*cis* isomer was separated from *trans* isomers and other impurities by silver resin chromatography. Location and configuration of the double bonds and deuterium atoms were affirmed by nuclear magnetic resonance and mass spectrometry.
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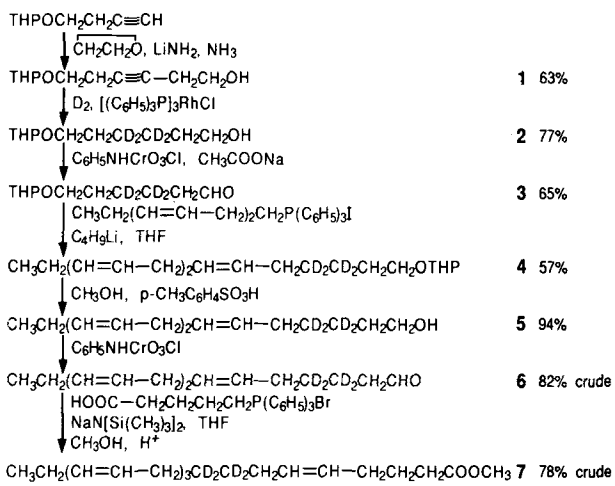
For studies of incorporation, elongation and desaturation of fats in humans and animals (1,2), we have synthesized various monoenoic (3), dienoic (4) and tri- and tetraenoic (5–7) fatty acid esters labeled with deuterium in various positions. In this paper we describe the synthesis of methyl 5,11,14,17-eicosatetraenoate-8,8,9,9-d_4 (5,11,14,17-20:4-d_4) which is potentially an intermediate in the conversion of linolenate (9,12,15-18:3) to eicosapentaenoate (5,8,11,14,17-20:5). The 5,11,14,17-20:4-d_4 has been synthesized to study the proposed pathway (8) that involves the action of a Δ8 desaturase. The results of the biochemical investigation will be published separately.

RESULTS AND DISCUSSION

The original plan was to generate methyl 5,11,14,17-20:4-d_4 by Wittig coupling of 3,6-nonadienyltriphenylphosphonium iodide with methyl 11-oxo-5-undecenoate-8,8,9,9-d_4. The synthesis of the nine carbon phosphonium salt has been described previously (6,7). An improved preparation of 1-bromo-2-pentyne, the first intermediate in the synthesis of the phosphonium salt, is presented in this paper. The required aldehyde ester was to be obtained by oxidation of methyl 11-hydroxy-5-undecenoate-8,8,9,9-d_4. We thought this hydroxy ester should be accessible through Wittig reaction of 3,3,4,4-tetradeutero-6-hydroxyhexyltriphenylphosphonium iodide and methyl 5-oxopentanoate. However, the reaction did not yield the desired Wittig coupling product. A hydroxyphosphonium salt reacts with a simple aldehyde to give the expected Wittig coupling product but, according to Ohta *et al.* (9), the principal product (about 35% yield) of the reaction of a hydroxyphosphonium salt and an aldehyde ester is a macrocyclic lactone rather than the Wittig coupling product. In an effort to avoid this unwanted reaction, we prepared the tetrahydropyranyl (THP) ether of 6-hydroxyhexyltriphenylphosphonium bromide but were unable to obtain it in crystalline form. We were unsuccessful in our attempts to isolate a Wittig product from the reaction between the viscous phosphonium salt and the five-carbon aldehyde ester.



SCHEME 1

We then decided to approach the synthesis from the other end of the molecule (Scheme 1). This would involve coupling, *via* a Wittig reaction, 3,6-nonadienyltriphenylphosphonium iodide with the THP ether of 6-hydroxyhexanal-3,3,4,4-d_4, **3**, to yield, after hydrolysis, 6,9,12-pentadecatrienol-3,3,4,4-d_4, **5**.

For the synthesis of compound **3**, we condensed the THP ether of 3-butynol with ethylene oxide in the presence of lithium amide in liquid ammonia to give the mono-THP ether of 3-hexyne-1,6-diol, **1**. This compound was treated with deuterium gas in the presence of *tris*-(triphenylphosphine)chlororhodium to give the mono-THP ether of 1,6-hexanediol-3,3,4,4-d_4, **2**. Oxidation of this intermediate with pyridinium chlorochromate in the presence of sodium acetate (10) produced the THP ether of 6-hydroxyhexanal-3,3,4,4-d_4, **3**. Compound **3** was coupled with 3,6-nonadienyltriphenylphosphonium iodide and butyl lithium to give the THP ether of pentadecatrienol-3,3,4,4-d_4, **4**, which was then hydrolyzed to **5**. The pentadecatrienol-d_4, **5**, could be converted to the phosphonium iodide through the bromide. The phosphonium iodide could then be coupled with methyl 5-oxopentanoate to give 20:4-d_4. Alternatively, compound **5** could be oxidized to the corresponding aldehyde and coupled with 4-carboxybutyltriphenylphosphonium bromide (which is commercially available) to give 20:4-d_4. We chose the latter route. Accordingly, pentadecatrienol-d_4, **5**, was oxidized to pentadecatrienal-d_4, **6**, with pyridinium chlorochromate. With unsubstituted phosphonium salts, the Wittig reaction with butyl lithium as base yields about 10–15% *trans* in the coupled product. With carboxybutyltriphenylphosphonium bromide and nonanal we found that the Wittig reaction with butyl lithium gave about 30% *trans*

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Abbreviations: DHP, dihydropyran; EE, diethyl ether; GC, gas chromatography; NMR, nuclear magnetic resonance; PE, petroleum ether (35–60°C); *p*-TSA, *p*-toluenesulfonic acid; THF, tetrahydrofuran; THP, tetrahydropyranyl.

isomer, and the yield was poor due to the formation of the aldol condensation product from the nonanal. We investigated other bases and settled on sodium *bis*(trimethylsilyl)amide (11) because it gave the desired product with only about 10% *trans*, and without the formation of the aldol product. Therefore we used sodium *bis*(trimethylsilyl)amide to condense pentadecatrienal-d_4, **6**, with 4-carboxybutyltriphenylphosphonium bromide to give compound **7**. The all-*cis* isomer of **7** was isolated on a 75% Ag/Na XN1010 column using 2–5% concentrations of acetonitrile in methanol as eluant.

EXPERIMENTAL PROCEDURES

Reagents. Butyl lithium, triphenylphosphine, dihydropyran, 4-carboxybutyltriphenylphosphonium bromide, 3-butynol, sodium *bis*(trimethylsilyl)amide and pyridinium chlorochromate were obtained from Aldrich Chemical Company (Milwaukee, WI). 2-Pentynol was purchased from Farchan Laboratories (Gainsville, FL), and *tris*(triphenylphosphine)chlororhodium was obtained from Strem Chemicals (Newburyport, MA). Silica gel (60–200 mesh) and Florisil (100–200 mesh) were purchased from J.T. Baker (Jackson, TN), and Silica Gel 60 A (70–230 mesh) was from American Scientific Products (McGaw Park, IL).

Methods. A 30 m × 0.25 mm SP2340 fused silica capillary column (Supelco, Inc., Bellefonte, PA) was used for analyzing binary mixtures of geometric isomers. For other analyses, a 6 ft × 4 mm column packed with 3% EGSS-X on 100/120 GasChrom Q or a 5 m × 0.53 mm HP-1 column (Hewlett-Packard Co., Avondale, PA) was employed.

^{13}C Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker (Billerica, MA) WM 300 WB pulsed Fourier transform spectrometer operating at 75.5 MHz. Typically, 2500 transients were collected from solutions in $CDCl_3$, which served as both internal lock and secondary reference, using 5-mm tubes. Sweep widths of 200 ppm and 8 K real data points limited acquisition time to 0.54 s and were used to obtain chemical shift values within ± 1.85 Hz, *i.e.*, ±0.05 ppm. A pulse width of 3 μs (40°) was employed with no delay between pulses. Decoupling power was held to *ca.* 1 W to provide adequate broadband decoupling power while minimizing sample heating (27°C probe temperature). The signal from carbons bearing two deuterium atoms or one deuterium atom and a double bond is diminished to such an extent that it is usually not detected.

Mass spectra were obtained on a Finnigan (San Jose, CA) 4500 mass spectrometer using isobutane chemical ionization with data processing of the isotope distribution against standards (12).

The macroreticular resin used for the separations was Rohm and Haas (Philadelphia, PA) XN1010 sulfonic acid resin ground to the mesh size indicated. Preparation of the silvered columns has been described (13–15).

Improved preparation of 1-bromo-2pentyne. Triphenylphosphine (171.5 g, 655 mmol) was dissolved in methylene chloride (325 mL) in a 1-L, three-necked flask equipped with a mechanical stirrer, a low temperature thermometer and a burette or dropping funnel. A slow stream of N_2 was maintained through the apparatus as it was cooled in an ice bath. Bromine (102.8 g, 33.2 mL, 643 mmol) was added dropwise over 50 min while the temperature was kept between 3 and 15°C by intermittent cooling. 2-Pentynol (50.1 g, 595 mmol) in CH_2Cl_2 (50 mL) was added to the off-white slurry over 30 min at 4 to 8°C. The ice bath was removed and 15 min later petroleum ether (PE) (400 mL) was added. The mixture was filtered with suction and the precipitate was washed with PE (2 × 100 mL). A column (3 × 50 cm) was packed with Baker silica gel (100 g), and 900 mL of solution was passed through the dry column followed by two 250 mL portions of PE. Solvent was removed from the eluate on a rotary evaporator at about 15°C and about 100 torr to yield 103 g of residue. The residue was distilled through a jacketed Vigreux column to yield the title compound (77.59 g, 88.7% yield, 98% pure by gas chromatography (GC) b.p. 63–80°C at 90 torr). In contrast to previously described preparations, this sample of 1-bromo-2-pentyne was not a lachrymator.

Preparation of 6-(2-tetrahydropyranyloxy)-3-hexynol (**1**) (16). Liquid ammonia (*ca.* 400 mL) was charged to a 1-L, three-necked flask equipped with a mechanical stirrer and a dry ice-cooled condenser and surrounded by Vermiculite insulating material. Ferric nitrate (0.4 g) was added followed by the slow addition of metallic lithium (2.62 g, 328 mmol). After the slurry had turned gray, a solution of the THP ether of 3-butynol (45.5 g, 295 mmol) in diethyl ether (EE) (25 mL) was added. Liquid ethylene oxide (35.28 g, 40 mL, 802 mmol) was added in one portion and stirring was continued for 8 h. The Vermiculite insulating material was removed, and the ammonia was permitted to vent overnight through a tube containing KOH pellets. The next morning, aqueous NH_3 (5 mL) and water (100 mL) were added. The reaction mixture was extracted with EE (1 × 100, 2 × 50 mL), and the combined EE layers were washed with saturated NaCl solution (100 mL) and dried (Na_2SO_4). Distillation through a jacketed Vigreux column gave a forerun (5.12 g) b.p. 45–80°C at 0.35 torr containing principally unreacted THP ether of 3-butynol and then the title compound, **1**, (36.53 g, 62.6% yield, 94% pure by GC) b.p. 93–130°C at 0.3 to 0.5 torr. ^1H NMR ($CDCl_3$; ppm): δ 1.41–1.73 (*m*, 6H, $CH_2CH_2CH_2$), 2.28-2.40 (*m*, 4H, $CH_2C{\equiv}CCH_2$), 2.76 (*t*, 1H, OH), 3.39–3.48 (*m*, 2H, CH_2O), 3.53–3.60 (*m*, 2H, $THPOCH_2$), 3.66–3.78 (*m*, 2H, CH_2OH), 4.54 (*m*, 1H, OCHO). ^{13}C NMR (ppm): C-1, 61.0; C-2, 22.9; C-3, 77.7; C-4, 78.9; C-5, 20.0; C-6, 65.8; THP group: C-2, 98.6; C-3, 30.4; C-4, 19.3; C-5, 25.2; C-6, 62.1.

Preparation of 6-(2-tetrahydropyranyloxy)hexanol-3,3,4,4-d_4, (**2**). Compound **1** (26.1 g, 132 mmol) in benzene (1 L) was treated with deuterium gas in the presence of *tris*-(triphenylphosphine)chlororhodium (3 g) in the manner previously described (3). Benzene was removed on the rotary evaporator, and the red liquid remaining was diluted with PE (100 mL), filtered and passed through a column (3 × 50 cm) containing Silica Gel 60 (100 g) in PE. Elution with increasing concentrations of EE in PE (0 to 50%) yielded fractions which were combined (26 g) and distilled through a jacketed Vigreux column to yield a forerun (0.39 g), b.p. 50–100°C at 0.5 torr containing all the impurities and a main fraction (20.84 g, 76.8% yield), b.p. 120–123°C at 0.6 torr containing the title compound, **2**, (98% pure by GC). Deuterium distribution: 2.3% d_3, 96.7% d_4, 1.0% d_5, 0.1% d_6. ^1H NMR ($CDCl_3$; ppm): δ 1.44–1.76 (*m*, 10H, $CH_2CH_2CH_2$ and $CH_2CD_2CD_2CH_2$), 2.08 (*s*, 1H, OH), 3.30–3.37 (*m*, 2H, CH_2O), 3.54–3.58 (*m*,

2H, THPOCH_2), 3.64–3.72 (*m*, 2H, CH_2OH), 4.51 (*m*, 1H, OC*H*O). ^{13}C NMR (ppm): C-1, 62.6; C-2, 32.3; C-5, 29.3; C-6, 67.4; THP group: C-2, 98.7; C-3, 30.6; C-4, 19.6; C-5, 25.4; C-6, 62.3.

Preparation of 6-(2-tetrahydropyranyloxy)hexanal-3,3,4,4-d_4, (3). Pyridinium chlorochromate (38.9 g, 183 mmol) and sodium acetate (3 g, 36.6 mmol) were suspended in methylene chloride (170 mL) in a 500-mL, three-necked flask equipped with a mechanical stirrer and a thermometer. A stream of nitrogen was maintained through the apparatus. A solution of compound 2 (18.8 g, 91.3 mmol) in CH_2Cl_2 (10 mL) was added in one portion. The reaction mixture turned black, and the mildly exothermic reaction was maintained between 25 and 30°C by intermittent use of an ice bath. Ninety minutes later, EE was added to the stirred mixture. The EE layer was decanted, and the black residue was stirred with two fresh portions (100 mL each) of EE and decanted. The combined EE layers were passed through a column containing Florisil (40 g) to yield a pale green liquid (17.83 g). This material may be used without further purification or it may be passed through a column (3 × 50 cm) containing Silica Gel 60 (100 g) in PE. The title compound, 3, (12.13 g, 65% yield, 89% pure by GC) elutes with mixtures containing up to 15% EE in PE. ^{1}H NMR ($CDCl_3$; ppm): δ 1.43–1.78 (*m*, 8H, CH_2CH_2CH_2 and CH_2CD_2), 2.37 (*s*, 2H, CH_2CHO), 3.28–3.47 (*m*, 2H, CH_2O), 3.64–3.83 (*m*, 2H, THPOCH_2), 4.49–4.51 (*m*, 1H, OC*H*O), 9.70–9.71 (*t*, 1H, C*H*O). ^{13}C NMR (ppm): C-1, 202.5; C-2, 43.5; C-5, 29.1; C-6, 67.1; THP group: C-2, 98.9; C-3, 30.7; C-4, 19.6; C-5, 25.4; C-6, 62.3.

Preparation of 2-(3',3',4',4'-tetradeutero-6',9',12'-pentadecatrienyloxy)tetrahydropyran, (4). 3,6,-Nonadienyltriphenyl phosphonium iodide (33.5 g, 65.4 mmol) was slurried in tetrahydrofuran (150 mL) in a 500-mL, three-necked flask equipped with a mechanical stirrer, a thermometer and a $CaCl_2$ drying tube. While a stream of nitrogen was maintained through the apparatus, the slurry was cooled to 5°C, and butyl lithium (2.5 M, 30 mL, 75 mmol) was added. A dark red solution was formed. Fifteen minutes later, compound 3 (12.13 g, 59.5 mmol) in tetrahydrofuran (THF) (15 mL) was added. The color of the reaction remained red due to the presence of excess phosphonium salt. The ice bath was removed, and 1 h later GC analysis of a sample of the reaction mixture (after reaction with saturated NaCl solution) showed absence of aldehyde and presence of a new component. Thirty minutes later saturated NaCl solution (100 mL) was added and the layers were separated. Solvent was removed from the organic layer on the rotary evaporator to give a liquid and solid (38.7 g). This mixture was extracted with PE (4 × 50 mL), and the combined PE layers were dried (Na_2SO_4). After removal of the drying agent and solvent, the residue was placed on a column (3 × 50 cm) containing silica gel 60 (100 g) in PE. The title compound, 4, (13.44 g, 57% yield, 90% pure by GC) was obtained by elution with PE containing 2 or 5% diethyl ether. ^{1}H NMR ($CDCl_3$; ppm): δ 0.96 (*t*, 3H, CH_3), 1.49–1.80 (*m*, 6H, CH_2CH_2CH_2), 2.01–2.08 (*m*, 4H, C=CH-CH_2), 2.78 (*t*, 4H, C=CHCH_2CH=C), 3.32–3.39 (*m*, 2H, OCH_2), 3.68–3.75 (*m*, 2H, THPOCH_2), 4.54–4.56 (*m*, 1H, OC*H*O), 5.27–5.41 (*m*, 6H, *H*C=C*H*). ^{13}C NMR (ppm): C-1, 67.5; C-2, 29.4; C-5, 26.9; C-6, 130.1; C-7, 127.7; C-8, 25.6; C-9,10, 128.2; C-11, 25.5; C-12, 127.0; C-13, 131.9; C-14, 20.5; C-15, 14.2; THP group: C-2, 98.7; C-3, 30.7; C-4, 19.6; C-5, 25.5; C-6, 62.2.

Preparation of 6,9,12-pentadecatrienol-3,3,4,4-d_4, (5). Compound 4 (4.55 g, 14.7 mmol) was dissolved in methanol (125 mL). *p*-Tbluenesulfonic acid (0.5 g) was added, and the flask was flushed with nitrogen and capped. The next morning, solid $NaHCO_3$ was added, and the solvent was removed on the rotary evaporator. The residue was dissolved in saturated $NaHCO_3$ solution (25 mL) and extracted into EE (2 × 25 mL). The EE solution was dried (Na_2SO_4), and after removal of the drying agent, there was obtained the title compound, 5, (3.11 g, 94% yield, 87% pure by GC). Deuterium distribution: 0.1% d_0, 0.4% d_2, 3.9% d_3, 93.4% d_4, 1.6% d_5, 0.3% d_6. ^{1}H NMR ($CDCl_3$; ppm): δ 0.93–0.98 (*t*, 3H, CH_3), 1.45–1.65 (*m*, 3H, O*H*, CH_2CH_2OH), 2.05 (*m*, 4H, CH_2C=C), 2.77 (*m*, 4H, C=CCH_2C=C), 3.60 (*t*, 2H, CH_2OH), 5.35 (*m*, 6H, C*H*=C*H*). ^{13}C NMR (ppm): C-1, 62.8; C-2, 32.4; C-5, 26.9; C-6, 130.0; C-7, 127.8; C-8, 25.6; C-9,10, 128.2; C-11, 25.5; C-12, 127.0; C-13, 131.9; C-14, 20.5; C-15, 14.2.

Preparation of 6,9,12-pentadecatrienal-3,3,4,4-d_4, (6). Pyridinium chlorochromate (40.7 g, 189 mmol) was suspended in methylene chloride (170 mL) in a 500-mL, three-necked flask equipped with a mechanical stirrer and a thermometer. A flow of nitrogen was maintained through the apparatus and 6,9,12-pentadecatrienol-d_4, 5, (21.4 g, 94.7 mmol) was added in one portion. The mildly exothermic reaction was controlled by intermittent cooling with an ice bath. One hour later, EE (100 mL) was added to the black viscous reaction mixture and stirring was continued for 10 min. Liquid was decanted and two fresh portions of EE (100 mL each) were stirred with the tar for about 10 min each and liquid was decanted. The combined decantates were passed through a column (2 × 30 cm) containing dry Florisil (40 g). The column was flushed with EE (50 mL), and the combined eluates were concentrated on the rotary evaporator to yield a slightly colored liquid (17.4 g, 82% crude yield, 84% pure by GC on HP-1). This liquid was used without further purification in the Wittig reaction with carboxybutyltriphenylphosphonium bromide. An analytical sample was obtained by elution through a SepPak (Waters, Milford, MA). ^{1}H NMR ($CDCl_3$; ppm) : δ 0.93–0.99(*t*, 3H, CH_3), 2.01–2.10 (*m*, 4H, CH_2C=C), 2.40 (*s*, 2H, CH_2CHO), 2.80 (*m*, 4H, C=CCH_2C=C), 5.30–5.40 (*m*, 6H, C*H*=C*H*), 9.74 (*t*, 1H, C*H*O). ^{13}C NMR (ppm): C-1, 202.4; C-2, 43.5; C-5, 26.7; C-6, 129.4; C-7, 128.0; C-8, 25.6; C-9,10, 128.4; C-11, 25.5; C-12, 127.0; C-13, 131.9; C-14, 20.5; C-15, 14.2.

Preparation of methyl 5,11,14,17-eicosatetraenoate-8,8,9,9-d_4, (7). Carboxybutyltriphenylphosphonium bromide (29.36 g, 66 mmol) was slurried in THF (125 mL) in a 500-mL, three-necked flask equipped with a mechanical stirrer, a thermometer and a septum inlet. While a stream of nitrogen was passed through the apparatus, sodium *bis*(trimethylsilyl)amide (1M, 133/mL) was added over a period of about 10 min. The mildly exothermic reaction was kept at 23–30°C by intermittent use of an ice bath. The septum inlet was replaced with a reflux condenser, and the red-orange reaction mixture was heated at the reflux temperature for 1.5 h. The reaction mixture was cooled in an ice bath, and compound 6 (14.8 g, 66 mmol) in THF (10 mL) was added and the color changed to tannish-orange. One hour later, water (25 mL) was added to the slurry. The resulting cherry red solution was cooled

in an ice bath as it was acidified with 5N H_2SO_4 (70 mL). The organic layer was separated, washed with saturated NaCl solution and dried (Na_2SO_4). Removal of the drying agent and solvent left an amber liquid (43.67 g). Methanol (200 mL) and concentrated sulfuric acid (1 mL) were added, and the reaction mixture was heated at reflux temperature for 2 h. The acid was neutralized with solid $NaHCO_3$. Solvent was removed on the rotary evaporator to give a mixture of solid and liquid (47 g). This was extracted with hexane (2 × 50 mL and 1 × 25 mL), and the solvent was removed to give a residue (23.15 g). The title compound was obtained by purification on a column (3 × 45 cm) containing Baker silica gel (100 g) in PE. Elution with up to 5% EE in PE gave a product (16.81 g, 78% crude yield, about 70% pure by GC on SP2340). The all-*cis* isomer was isolated by silver resin chromatography on a 62% Ag/Na XN1010 resin column using 2% acetonitrile in methanol as eluant. Deuterium distribution. 0.3%d_1, 0.7%d_2, 2.8%d_3, 94.8%d_4, 1.3%d_5, 0.2%d_6, 0.1%d_8 (Ave. No. deuteriums=3.97). ^{1}H NMR ($CDCl_3$; ppm): δ 0.96 (*t*, 3, CH_3), 1.67 (*m*, 2H, CH_2CH_2COO), 1.91-2.1 (*m*, 8H, $C{=}CCH_2$), 2.3 (*t*, 2H, CH_2COO), 2.78 (*m*, 4H, $C{=}CCH_2C{=}C$), 3.65 (*s*, 3H, OCH_3), 5.32-5.36 (*m*, 8H, $CH{=}CH$). ^{13}C NMR (ppm): C-1, 174.0; C-2, 33.4; C-3, 24.9; C-4,7 and 10, 26.5 or 26.9; C-5, 130.9; C-6, 128.4; C-11, 130.1; C-12, 127.7; C-13, 25.6, C-14,15, 128.2; C-16, 25.5; C-17, 127.1; C-18, 131.9; C-19, 20.5; C-20, 14.3. Chemical shift assignments are consistent with data published by Gunstone *et al.* (17) and Gunstone (18) for similar compounds.

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METHOD

The Enrichment of n-3 Polyunsaturated Fatty Acids Using Aminopropyl Solid Phase Extraction Columns

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A rapid, simple and reliable method is described for the preparation of concentrates of methyl or ethyl esters of n-3 polyunsaturated fatty acids by solid phase extraction using aminopropyl bonded silica columns. After applying mixtures of fatty acid esters in hexane, saturated and monounsaturated fatty acid esters are preferentially eluted with hexane whereas polyunsaturated fatty acids (PUFA) can subsequently be eluted with dichloromethane. Concentrates containing 80–90% n-3 PUFA can thus be obtained using fish oil fatty acids esters as a starting material.

Lipids 28, 51–54 (1993).

The concentration of polyunsaturated fatty acids (PUFA) can be achieved by utilizing differences in physical or chemical properties between saturated and unsaturated fatty acids. Low temperature crystallization, urea adduct fractionation and fractional distillation are all traditional methods used for the preparation of concentrates of n-3 PUFA from derivatives of fish oil fatty acids (1–4) and are best suited to large-scale preparations. Adsorption chromatography using layers or columns of silica gel impregnated with silver nitrate has been useful to isolate gram quantities of individual PUFA (5,6), whereas reversed-phase high-performance liquid chromatography (HPLC) has been applied for the same purpose on the milligram scale (7,8). More recently, planar thin-layer chromatography (TLC) using high-performance thin-layer silica gel has been used for the separation of C_{20} and C_{22} PUFA (9). The selective hydrolysis, by neutral lipases, of fish oil triacylglycerols, or of derivatives of their component fatty acids, has also been used to produce fractions enriched in n-3 PUFA (10). Used in conjunction with the above techniques, supercritical fluid carbon dioxide fractionation of esters of fish oil fatty acids has been utilized to produce highly enriched fraction of individual PUFA (11).

In the present report, a novel method is described for the isolation of PUFA concentrates from fish oil fatty acid methyl or ethyl esters by solid phase extraction using aminopropyl bonded silica.

MATERIALS AND METHODS

Materials. Aminopropyl columns (Bond-Elut) were purchased from Jones chromatography (Hengord, U.K.) and from J.T. Baker (Hayes, U.K.). Fish oil derived from sardines or anchovies was obtained from Seven Seas Ltd. (Hull, U.K.). All solvents were of HPLC grade and were purchased from Rathburn Chemicals (Walkerburn, U.K.). [1-^{14}C]18:0 (52.7mCi/mmol), [1-^{14}C]18:1n-9 (50.0mCi/mmol), [1-^{14}C]18:2n-6 (54.7mCi/mmol) and [1-^{14}C]18:3n-3 (53.9mCi/mmol), all as free acids, were purchased from Amersham International (Aylesbury, U.K.). Ecoscint A scintillation fluid was supplied by National Diagnostics (Manville, NJ).

Preparation of methyl and ethyl esters of fatty acids. Methyl esters of the fatty acids from fish oil were prepared by subjecting samples of the oil directly to acid-catalyzed transmethylation in methanol containing 1% sulfuric acid for 16 h at 50°C under nitrogen (12). Ethanol was substituted for methanol for the preparation of ethyl ester derivatives (12). [^{14}C]Fatty acid methyl esters (FAME) were prepared as described above by subjecting total lipid isolated from rat brain cell cultures that had been incubated with 1 μM/0.5 μCi of [1-^{14}C]18:0, [1-^{14}C]18:1n-9, [1-^{14}C]18:2n-6 or [1-^{14}C]18:3n-3 for 48h. Mixtures of saturated fatty acids (SFA) contained 85% 18:0, 7% 20:0 and 8% longer chain saturated fatty acids as [^{14}C]FAME. Mixtures of monounsaturated fatty acids (MUFA) contained 75% 18:1, 6% 20:1, 14% 24:1 and 5% > 24:1 as [^{14}C]FAME. Mixtures of n-6 PUFA contained 70% 18:2, 10% 20:3 and 20% 20:4 as [^{14}C]FAME, whereas n-3 PUFA contained 15% 18:3, 20% 20:5, 40% 22:5 and 25% 22:6 as [^{14}C]FAME.

Fatty acid methyl or ethyl esters were extracted from the reaction mixture using hexane/diethyl ether (1:1, vol/vol) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. After removal of the solvent by rotatory evaporation, the derivatives were redissolved in hexane containing 0.01% BHT and purified using 500 mg aminopropyl columns (Bond-Elut) (13). For this purpose, the columns were prewashed sequentially with 4 mL (7 bed volumes) hexane/glacial acetic acid (98:2, vol/vol) and 4 mL hexane before application of the fatty acid methyl or ethyl esters in hexane. Under these conditions the aminopropyl silica is protonated, and fatty acid esters are not retained on the column and are eluted using 4 mL hexane. Traces of free cholesterol, free fatty acids and triacylglycerols remain on the column (14) as do monohydroxy and monohydroperoxy fatty acids and polymeric material (unpublished results). To elute bound material, the columns were washed with 4 mL hexane/glacial acetic acid (98:2, vol/vol) followed by 4 mL hexane. The column could then be then be reused for the purification of fatty acid esters from the components described above.

Solid phase extraction. A 500 mg aminopropyl bonded silica column was prewashed using 4 mL dichloromethane and then 4 mL hexane. In these experiments, 5 mg of methyl or ethyl esters of fish oil fatty acids dissolved in 0.5 mL hexane were then applied to the column. Using a syringe attached to the column by an adapter, 4 mL of hexane (7 bed volumes) were slowly pushed through the column, and the eluting solvent collected in a test tube. Similarly, 4 mL of dichloromethane was applied to the

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Abbreviations: BHT, butylated hydroxytoluene; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TLC, thin-layer chromatography.

column, and the eluting solvent collected in a tube. The solvent was evaporated under a stream of nitrogen; the components present in the hexane and dichloromethane fractions were redissolved in hexane containing 0.01% BHT and analyzed by capillary gas-liquid chromatography (GLC) as described below. After elution with hexane and dichloromethane, the column was washed with 4 mL hexane before loading the next sample.

Quantification of [^{14}C] fatty acid methyl esters. Saturated, monounsaturated and polyunsaturated [^{14}C]-FAME (0.1 μC_1) were mixed separately with 2.5 or 15 mg of fish oil FAME and applied to aminopropyl columns dissolved in hexane according to the solid phase extraction procedure described above. The hexane and dichloromethane fractions were collected directly into scintillation vials, and the solvent removed under nitrogen. Scintillation fluid (2.5 mL) was added and the level of radioactivity determined by liquid scintillation counting using a Canberra Packard (Pangbourne, U.K.) Tricarb 2000CA liquid scintillation analyzer.

Gas-liquid chromatography (GLC). Methyl and ethyl esters of fatty acids were analyzed on a chemically bonded CP Wax 52CB fused silica capillary column (50 m × 0.34 mm i.d.) (Chrompack, London, U.K.) contained in a Canberra Packard 436 gas chromatograph equipped with on-column injection and a flame ionization detector, using hydrogen as carrier gas and a thermal gradient from 50° to 225°C (15). Individual methyl and ethyl esters were identified by comparison with authentic standards and those prepared from a well-characterized fish oil. Values are recorded as means ± SD for three separate experiments.

RESULTS AND DISCUSSION

When mixtures of [^{14}C]FAME were combined with fish oil FAME and applied to aminopropyl columns as described in the Materials and Methods, all (99.5 ± 1.5%) the radioactivity applied to the column was recovered in the eluting solvents. The recoveries of the various types of [^{14}C]FAME in the hexane and dichloromethane fractions are shown in Table 1 as a function of the amount applied to the column. The distribution patterns of radioactivity from ^{14}C-labelled, saturated, monounsaturated and polyunsaturated FAME in the two eluants were markedly different and were dependent on column loading. In addition, it was noted that the proportion of radioactivity recovered in the dichloromethane fraction was related to the degree of unsaturation of the FAME. Thus, 83% of n-6 [^{14}C]PUFA and 95% of n-3 [^{14}C]PUFA were recovered in the dichloromethane fraction at a column loading of 0.5% (wt/wt), whereas only 33% of n-6 [^{14}C]PUFA and 79% of n-3 [^{14}C]PUFA were recovered in the dichloromethane fraction using a column loading of 3% (wt/wt). The difference in recovery between n-6 and n-3 [^{14}C]PUFA correlates with overall degree of unsaturation, an average of 2.5 and 5 double bonds for n-6 PUFA and n-3 PUFA, respectively.

The fatty acid composition of the fish oil employed in this study is typical of the starting material used in the preparation of PUFA concentrates or for the isolation of 20:5n-3 or 22:6n-3 as individual fatty acids (1). Of the total FAME, 41.3% were PUFA and 20:5n-3 and 22:6n-3 accounted for 20.4% and 8.9%, respectively. Saturated and monounsaturated fatty acids accounted for 29.1% and 29.6%, respectively, of total FAME with 14:0, 16:0, 16:1n-7 and 18:1n-9 being major components.

The maximum loading capacity of aminopropyl columns was found to be 1–4% by weight depending on the batch tested. When the basic solid phase extraction procedure was used at maximum loading capacity for the separation of the fish oil esters, the hexane and dichloromethane fractions contained 55 ± 3% and 45 ± 3%, respectively, of the fatty acid derivatives applied to the column. The fatty acid esters recovered in the two fractions were markedly different in composition (Table 2). SFA, MUFA and PUFA comprised 47.3, 42.7 and 10%, respectively, of the fatty acid esters in the hexane fraction. The corresponding values for the dichloromethane fraction were 2.6%, 8.7% and 88.7%, and this fraction contained almost all the 20:5n-3 and 22:6n-3 applied to the column. The proportions of individual PUFA, particularly those of the n-3 series, in the dichloromethane fraction were approximately double those in the original fish oil esters, and it was noted that the ratio of 20:5n-3 to 22:6n-3 was around 2 to 1 in both the original fish oil esters and the dichloromethane fraction. Fractions having the compositions presented in Table 2 could be obtained repeatedly using the same column up to 12 times without loss of function, and very similar results were found using four different batches of aminopropyl columns. Furthermore, no difference was observed between the methyl and ethyl ester derivatives of the fish oil fatty acids in terms of their chromatographic behavior in the procedures examined in this study.

A simple modification of the basic procedure using hexane/dichloromethane, (98:2, vol/vol) as an eluant and

TABLE 1

Recovery of [^{14}C]Fatty Acid Methyl Esters in Fractions Obtained by Solid Phase Extraction on Aminopropyl Bonded Silica[a]

	SFA		MUFA		n-6 PUFA		n-3 PUFA	
Loading (wt/wt)[b]	0.5	3	0.5	3	0.5	3	0.5	3
	Total radioactivity recovered (%)							
Hexane	75 ± 5	94 ± 2	51 ± 4	88 ± 2	17 ± 3	67 ± 5	5 ± 2	21 ± 4
Dichloromethane	25 ± 5	6 ± 2	49 ± 4	12 ± 2	83 ± 3	33 ± 5	95 ± 2	79 ± 4

[a]Values are expressed as percentage of total radioactivity recovered in each fraction and are means ± SD for three separate experiments. All the radioactivity applied to the columns was recovered either in the hexane fraction or in the dichloromethane fraction (*i.e.*, 99.5 ± 1.5%, n = 12). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[b]Column loading is expressed as a percentage aminopropyl weight.

TABLE 2

Fatty Acid Composition of Methyl Esters of Fish Oil and Fractions Obtained by Solid Phase Extraction on Aminopropyl Bonded Silica[a]

	Fish oil	Fraction	
		Hexane	Dichloromethane
	Total fatty acids (wt%)		
14:0	7.9 ± 0.2	13.0 ± 0.6	0.7 ± 0.4
16:0	18.0 ± 0.3	29.0 ± 2.1	1.7 ± 0.7
18:0	2.8 ± 0.2	4.6 ± 0.3	0.2 ± 0.2
20:0	0.2 ± 0.1	0.5 ± 0.2	0.0 ± 0.0
22:0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
24:0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
Total SFA	29.1 ± 0.7	47.3 ± 2.2	2.6 ± 0.5
16:1	11.3 ± 0.2	15.8 ± 1.6	4.6 ± 0.4
18:1	11.9 ± 0.3	17.8 ± 1.7	3.2 ± 0.9
20:1	2.6 ± 0.2	3.7 ± 0.7	0.4 ± 0.1
22:1	3.1 ± 0.2	3.9 ± 1.0	0.4 ± 0.1
24:1	0.6 ± 0.1	1.0 ± 0.1	0.1 ± 0.1
Total MUFA[b]	29.6 ± 0.9	42.7 ± 1.8	8.7 ± 1.1
16:2n-4	0.8 ± 0.1	0.6 ± 0.2	1.0 ± 0.1
16:3n-4	0.8 ± 0.1	0.5 ± 0.2	1.3 ± 0.1
16:4n-1	2.6 ± 0.1	0.7 ± 0.1	5.8 ± 0.2
18:2n-6	0.7 ± 0.1	0.9 ± 0.3	0.9 ± 0.2
18:3n-3	0.8 ± 0.2	0.5 ± 0.1	1.0 ± 0.1
18:4n-3	2.9 ± 0.2	0.8 ± 0.2	6.5 ± 0.3
20:4n-6	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.1
20.4n-3	0.5 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
20:5n-3	20.4 ± 0.4	3.7 ± 0.6	44.5 ± 1.4
21:5n-3	0.6 ± 0.1	0.2 ± 0.1	1.6 ± 0.1
22:5n-3	1.8 ± 0.2	0.3 ± 0.1	3.7 ± 0.2
22:6n-3	8.9 ± 0.3	0.7 ± 0.2	20.4 ± 1.0
Total PUFA	41.3 ± 1.3	10.0 ± 0.7	88.7 ± 1.8

[a]Values are means ± SD for three separate experiments. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[b]Includes all isomers; mainly 16:1n-7, 18:1n-9, 20:1n-9, 22:1n-11 and 24:1n-9. In these experiments 5 mg of fatty acid methyl ester was used, which represents a maximum column loading of 1% of aminopropyl weight.

subsequent elution of the column with dichloromethane yielded a fraction containing only PUFA but just 9 ± 2% of the fatty acid derivatives applied to the column. However, 20:5n-3 and 22:6n-3 each accounted for 45% of the total and 16:4n-1 (3%), 18:4n-3 (2.3%), 20:4n-3 (0.4%), 20:4n-6 (0.4%), 21:5n-3 (1.5%) and 22:5n-3 (2.4%) accounting for the remaining 10%. The presence of 22:6n-3 and 20:5n-3 in equal proportion reflects the stronger retention of 22:6n-3 than 20:5n-3 by the adsorbent since the ratio of 20:5n-3 to 22:6n-3 in the starting fish oil esters was approximately 2 to 1.

The above data show that aminopropyl columns are capable of concentrating PUFA from mixtures of fatty acid esters by retaining them on the adsorbent when the column is eluted with hexane. Aminopropyl bonded silica is an adsorbent that exhibits strong hydrogen bonding properties and functions also as a weak anion exchanger (13,16). PUFA exhibit strong dipoles at methylene-interrupted carbon atoms (17), and the formation of dipole-dipole interaction between the hydrogen atoms of these carbon atoms and aminopropyl bonded silica may form the basis of the interaction between PUFA and the aminopropyl groups. SFA and MUFA exhibit weaker dipoles than PUFA (17) and therefore are likely to bind to aminopropyl bonded silica to a lesser extent than PUFA. Since hexane is a nonpolar solvent and has an eluotropic strength of zero (16), it probably does not interfere with interactions based on dipole-dipole interaction between PUFA and the aminopropyl groups of the bonded silica, whereas saturated and monounsaturated fatty acids, being less strongly bonded to the adsorbent, can be eluted using hexane.

The capacity of the adsorbent is low under the conditions used here but the procedure can be easily scaled up by increasing the quantity of adsorbent used for extraction. Furthermore, the characteristics of the bonded silica permit rapid solvent flow through the adsorbent bed under conditions of minimal pressure (10-15 psi) (13). Although PUFA can be concentrated from fatty acid mixtures by other means, including low temperature crystallization, urea adduct fractionation and fractional distillation (1-4), these techniques usually have inherent disadvantages such as the requirement for sophisticated equipment, large volumes of solvent, or low yields. Furthermore, there is a risk of structural modification of PUFA during fractional distillation. Biotechnological procedures involving fungal lipases may be prohibitory in cost. Therefore, procedures based on the method described here, using large reusable columns packed with aminopropyl bonded silica, may provide an alternative to traditional methods for the preparation of PUFA concentrates. On a small scale, the method may prove most useful as a rapid method of enriching fatty acid mixtures in PUFA before further separation by other techniques such as silver nitrate chromatography or HPLC (5-8). Indeed, given further development, aminopropyl HPLC bonded silica (18) may ultimately provide analytical capabilities similar to silver nitrate chromatography or reversed-phase HPLC for the analysis of fatty acid derivatives.

In conclusion, the procedure described here allows the simple, rapid and reproducible concentration of methyl or ethyl esters of n-3 PUFA from natural mixtures of fatty acids. The procedure may lend itself to the large-scale preparation of high quality concentrates of n-3 PUFA for applications in human nutrition (19).

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Multigram Synthesis of 1-Alkylamido Phospholipids

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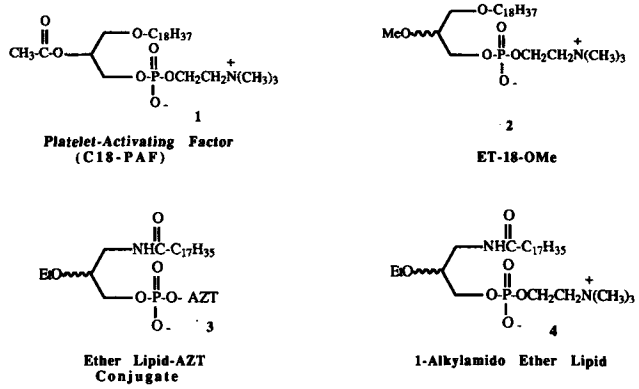
Phospholipids containing a 1-alkylamido linkage have shown promising *in vitro* neoplastic cell growth inhibitory properties and anti-human immunodeficiency viral activity. We have synthesized a series of alkylamido ether lipid analogues on a milligram scale for initial evaluation, but for further *in vivo* testing of these bioactive phospholipids, synthesis on a larger scale is required. The multigram synthesis of 1-alkylamido ether phospholipids was accomplished by modifying reaction conditions in the amidation step and changing reagents and solvent systems in both the detritylation and phosphorylation steps. This was most crucial in the phosphorylation step, where in the multigram synthesis 2-bromoethyl dichlorophosphate in diethyl ether/tetrahydrofuran (7:3, vol/vol) gave much improved yields as compared to the 2-chloro-2-oxo-1,3,2-dioxaphospholane reagent. The modifications also resulted in a product that could be more easily purified in sufficient quantities for use in *in vivo* inhibition studies.
Lipids 28, 55–57 (1993).

Over the past decade, it has become apparent that some classes of phospholipids may play a significant role in inflammatory processes, in cellular signalling events, in tumor growth inhibition, and in the processing of human immunodeficiency virus (HIV). For example, platelet-activating factor (1, Scheme 1) is a well-documented mediator of inflammatory processes and is involved in various other biological processes as well (1,2). Other alkyl ether lipids and their analogues (2–4, Scheme 1) have been shown to be potent, biologically active molecules (2–5) with the ability to modify biological responses through their action at the cell membrane level (3,4) or by modifying signal transduction pathways (5). The 1-alkylamido types of lipids (*e.g.*, 4, Scheme 1) have shown promise as neoplastic cell growth inhibitors (4) and anti-HIV agents (6,7) based on their *in vitro* activities. Further evaluation of these ether lipids required the preparation of the compounds in multigram quantities. As is often the case when scaling up a procedure that is adequate for synthesis on the milligram scale, problems arose in both the reaction and purification steps. Specific improvements were realized in the amidation reaction, in the detritylation of the 1-alkylamido-2-alkyl-3-tritylpropanediol intermediates, and in the subsequent phosphorylation step. The present paper describes the problems associated with and the alternative methods developed for the multigram synthesis of 1-alkylamido ether lipids.



SCHEME 1

RESULTS AND DISCUSSION

The multigram synthesis of four phospholipids containing 1-alkylamido linkages was completed as shown in Scheme 2. The synthesis of these compounds on a multigram scale required several modifications of our previously published procedure (4) for the synthesis of milligram quantities of these alkylamido lipids.

Using commercially available (±)-3-amino-1,2-propanediol 5 and the appropriate acid chloride (palmitoyl or stearoyl), the amidation reaction (4) was carried out in a larger volume of pyridine, omitting *N,N*-dimethylformamide. The amidation reaction thus proceeded smoothly, which appears to be an improvement over published procedures (8,9). Our previous method produced a significant amount of ester and ester/amide by-products that were difficult to remove during purification on the larger scale. A more dilute reaction solution resulted in a mixture that was more easily stirred, produced less ester by-product, and was easier to purify (one precipitation from isopropanol) than previously reported (4). The alkylamidopropanediol 6 formed by the new procedure was of higher purity, as judged by the 300 MHz ^1H nuclear magnetic resonance (NMR) data (*i.e.*, no signal was found that corresponded to ester α-methylene protons), and based on the higher melting point (106–107°C *vs.* 96–98°C, ref. 4). The tritylation was carried out using *N,N*-dimethylaminopyridine (DMAP) in CH_2Cl_2 to yield the desired product 7 which did not result in an improvement over the previously published procedure (4). The alkylation of the 2-hydroxyl group to produce 8 was performed as before using NaH and either methyl or ethyl iodide in tetrahydrofuran (THF).

Use of *p*-toluenesulfonic acid in CH_2Cl_2/CH_3OH (10) rather than BF_3/MeOH for removal of the trityl group also simplified the purification. The trityl methyl ether formed with the former reagent is more easily removed than the trityl alcohol produced by the latter, either by recrystallization of the product, alcohol 9, from hexane or by chromatography with the solvent CH_2Cl_2/MeOH (95:5, vol/vol) or hexane/ethyl acetate (2:1, vol/vol). The yields were also generally higher using the new reagent

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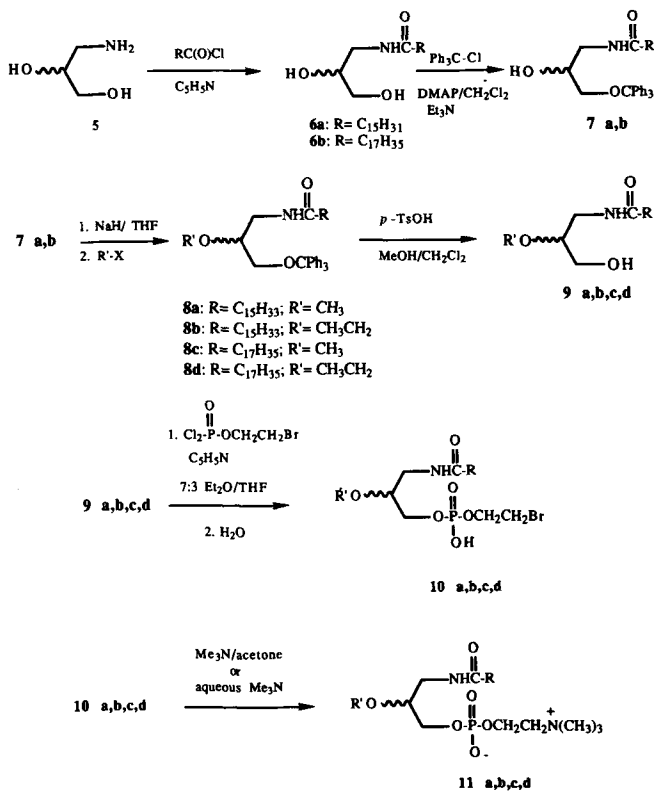
Abbreviations: CEP-Cl, cyclic enediol phosphochloridate; DMAP, *N,N*-dimethylaminopyridine; DMF, dimethylformamide; HIV, human immunodeficiency virus; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; TLC, thin-layer chromatography.

(90% *vs.* 50% for the 3-hexadecylamido-2-methoxypropanol).

A final major problem was the phosphorylation of the 1-alkylamido-2-alkylpropanol intermediate. Different phosphorylation reagents have been used in attempts to synthesize the alkylamidolipids: (i) $POCl_3$, (ii) 1,2-dimethylethenylene phosphochloridate (cyclic enediol phosphochloridate, CEP-Cl), (iii) 2-chloro-2-oxo-1,3,2-dioxaphospholane, or (iv) 2-bromoethyl dichlorophosphate. As noted previously (4,9,11), $POCl_3$ proved unsuitable in the presence of a variety of bases (Et_3N, C_5H_5N, or 2,6-lutidine) and resulted in polyphosphorylation and/or decomposition of the starting material. Previously, the appropriate phosphocholine **11** was synthesized on a milligram scale by treating the 3-alkylamidoether propanol intermediate **9** with 2-chloro-2-oxo-1,3,2-dioxaphospholane followed by treatment with condensed trimethylamine. However, in the multigram synthesis, this procedure gave poor results with milligram yields from 2–3 g of starting material.

Previous attempts to phosphorylate these 3-alkylamido ether lipids with 2-bromoethyl dichlorophosphate were unsuccessful due to difficulties in finding appropriate solvents (9,12–14). The alkylamido ether lipids are much less soluble in less polar solvents, as compared to the corresponding acyl, thioether or ether analogues. The starting material was not soluble in Et_2O, and solvents such as $CHCl_3$ have been shown to lead to chlorinated and polyphosphorylated by-products (14). However, the alcohol starting material was soluble in a mixture of Et_2O/THF (7:3, vol/vol), and the product (bromoethyl phosphochloridate **10**) precipitated out of the reaction mixture before further side reactions could occur, analogous to the approach described by Hansen *et al.* (14). It was absolutely critical to purify the intermediate bromoethyl phosphates to remove any traces of pyridine. The presence of pyridine in the amination reaction resulted in some pyridinium analogue formation, as indicated by ^1H NMR and the appearance of a fluorescent product peak in thin-layer chromatography (TLC). Therefore, all bromoethyl-phosphates were purified by chromatography prior to amination with either anhydrous or aqueous trimethylamine to produce the desired phosphocholine ether lipids **11** from the 3-alkylamido glycerols **9** in gram quantities in yields of 23–40%. The yields for the phosphorylation of multigram quantities using the bromoethyl dichlorophosphate/Me_3N procedure were similar to those reported for the milligram synthesis *via* 2-chloro-2-oxo-1,3,2-dioxaphospholane.



SCHEME 2

EXPERIMENTAL PROCEDURES

(±)-3-Amino-1,2-propanediol, palmitoyl chloride, stearoyl chloride, chlorotriphenylmethane (trityl chloride), 4-DMAP, 80% sodium hydride (oil dispersion), methyl iodide, ethyl iodide, *p*-toluenesulfonic acid monohydrate, and aqueous (40%) trimethylamine were used as obtained from the supplier (Aldrich, Milwaukee, WI). Anhydrous trimethylamine was condensed in a coldtrap (liquid nitrogen) immediately prior to use. Pyridine and triethylamine (Et_3N) were distilled from KOH, and THF was distilled from $LiAlH_4$. All reactions requiring anhydrous or inert conditions were performed under a positive pressure of dry nitrogen. 2-Chloro-2-oxo-1,3,2-dioxaphospholane (15,16) and 2-bromoethyl dichlorophosphate (12) were prepared according to literature procedures and freshly distilled before use. Chromatographic purifications were done using silica gel 60 (230–400 mesh; Merck, Darmstadt, Germany) and the indicated solvent systems. TLC was done on Whatman MK6F plates (Maidstone, England) and fractions were visualized by either spraying with sulfuric acid/dichromate or phosphomolybdate reagent followed by heating. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H NMR spectra were determined on a Bruker (Karlsruhe, Germany) AC-300 300-MHz spectrometer or a Varian (Palo Alto, CA) FT-80A spectrometer.

(±)-3-Hexadecanamido-1,2-propanediol (**6a**). To a mechanically stirred solution of (±)-3-amino-1,2-propanediol **5** (33.2 g, 0.36 mol) in 200 mL pyridine was added 100 g (0.36 mol) palmitoyl chloride over 1.5 h at room temperature. After addition, the reaction was stirred for three hours, and the mixture was poured onto an ice/water mixture with vigorous stirring. The solid crude product was filtered on a coarse sintered glass filter and the filtrate discarded. The precipitate was dissolved in 2 L of solvent ($CHCl_3$/MeOH, 2:1, vol/vol) and extracted successively with 2 × 200 mL 10% HCl, 3 × 200 mL 10% NaOH, and 2 × 200 mL 10% HCl, using MeOH to break the emulsions that formed. The nonaqueous layer was dried over anhydrous $MgSO_4$, filtered and solvents were removed with a rotary evaporator. The resulting solid was dissolved in 1.8 L of warm isopropanol, decanted from a small amount of insoluble material, and left at room temperature. The precipitated product was filtered and the filtrate placed in a −5° coldroom to precipitate a second crop of

product. The product **6a** was dried under vacuum over P_2O_5 to yield 85.3 g (72%) of a white powder (m.p. 106–107°C; lit. ref. 4, 96–98°C) that was pure by TLC (R_f 0.43, $CHCl_3$/MeOH, 9:1, vol/vol). The same procedure was followed to synthesize the octadecanamido analogue **6b** in 68% yield (m.p. 111.0–111.5°C; lit. ref. 4, 104–106°C).

(±)-3-Hexadecanamido-1-(triphenylmethoxy)-2-propanol (**7a**). To a slurry of the amidodiol **6a** (25 g, 0.076 mol) in 750 mL CH_2Cl_2 was added triethylamine (23 g, 0.228 mol) and 4-DMAP; 0.375 g, 3 mmol). The mixture was heated to 35°C, trityl chloride (23 g, 83.5 mmol) was added in one aliquot, and the reaction stirred overnight. TLC of an aliquot in hexane/ethyl acetate (2:1, vol/vol) showed the presence of starting material; so the reaction was heated at 60°C for 24 h. The reaction was cooled to room temperature and the solvents removed with a rotary evaporator, resulting in a viscous oil that solidified on standing. The residue was taken up in 1 L CH_2Cl_2 and extracted with 3 × 250 mL cold 10% HCl, 250 mL saturated NaCl, and dried over $MgSO_4$. The drying agent was filtered, the solvent removed on a rotary evaporator, the residue taken up in 800 mL MeOH and placed in a cold room at −5°C. The precipitate was filtered and dried as before to yield 54.2 g of product **7a** that was identical to the previously prepared material (4). The same procedure was utilized to synthesize the octadecanamido analogue **7a** in 72% yield, resulting in 43 g of product (m.p. 86–88°C).

(±)-3-Hexadecanamido-2-methoxy-1-(triphenylmethoxy)-propane (**8a**). The compound was synthesized as before (4) from 28.2 g (0.049 mol) of **7a** and purified by flash chromatography on 300 g silica gel (hexane/ethyl acetate, 2:1, vol/vol) to yield 18.5 g (65%) of product **8a**. The remaining intermediates were synthesized by the same procedure and were either used without further purification (**8b, 8c**) or purified by the same chromatographic procedure (**8d**, 78%).

(±)-3-Hexadecanamido-2-methoxy-1-propanol (**9a**). To 160 mL CH_2Cl_2/MeOH (5:1, vol/vol) was added 18.5 g (31.6 mmol) of the tritylated compound **8a** and 1.0 g of *p*-toluenesulfonic acid monohydrate. The reaction was stirred at room temperature for 15 h at which time TLC in hexane/ethyl acetate (2:1, vol/vol) showed no remaining starting material. The acid catalyst was neutralized with saturated sodium bicarbonate solution (~50 mL), and 50 mL H_2O was added. The nonaqueous layer was separated and extracted with 3 × 100 mL H_2O, the nonaqueous layers combined and extracted again with 100 mL H_2O and dried over anhydrous Na_2SO_4. Solvents were removed with a rotary evaporator and the crude solid residue was purified by flash chromatography on 300 g of silica gel in CH_2Cl_2/MeOH (95:5, vol/vol) to give 9.8 g (91%) of product **9a** (m.p. 67–68°C; lit. ref. 4, 61–62°C). The other analogues (**9b–9d**) were prepared in 45–63% yields using the same procedure.

(±)-3-Octadecanamido-2-methoxypropyl-1-phosphocholine (**11c**). To 150 mL anhydrous Et_2O/THF (7:3, vol/vol), cooled to 0°C in an ice bath under a positive atmosphere of dry nitrogen, were added 2.2 mL (0.016 mol) of freshly distilled 2-bromoethyl dichlorophosphate (12), followed by 6.5 mL pyridine. The alcohol **10c** (2.0 g, 0.0054 mol) was added to the reaction flask and the mixture warmed to room temperature and then heated to gentle reflux for 4 h. The solution was cooled to 4°C in an ice bath, 6 mL H_2O was added and stirred for 30 min. Solvents were removed with a rotary evaporator to give a semisolid that was dissolved in $CHCl_3$/MeOH (5:1, vol/vol). The solution was extracted with water, dried over sodium sulfate, filtered, and solvents were removed as before. Column chromatography on silica gel with a gradient of $CHCl_3$/MeOH (100:0 to 3:1, vol/vol) gave 600 mg of pure product and 1.6 g of impure product. The impure material was rechromatographed using $CHCl_3$/hexane (5:1, vol/vol), $CHCl_3$, and $CHCl_3$/MeOH (15:1 to 10:1, vol/vol) to give a further 1.0 g of pure bromoethylphosphate **10c**. The yield for **10c** was 52%, and the range for the other analogues was 37–47%.

The intermediate **10c** (1.4 g, 0.0025 mol) was dissolved in a solution of $CHCl_3$/*i*-PrOH/DMF (91 mL, 3:3:5, vol/vol/vol) and trimethylamine (18 mL, 40% aqueous) was added. The reaction mixture was heated to 65°C for 5 h and then cooled to room temperature before Ag_2CO_3 (1.2 g) was added. The mixture was heated for 1 h, cooled to room temperature, and filtered. Solvents were removed on a rotary evaporator, and the phospholipid was purified by column chromatography using $CHCl_3$/MeOH (10:1 to 3:1, vol/vol) followed by $CHCl_3$/MeOH/NH_4OH (75:25:5, vol/vol/vol) to give 1.0 g (76%) of pure product **11c**. The amination step for the other intermediates gave 47–63% yields. The overall yield of phospholipid **11c** from the alcohol **9c** was 40% and the range for the other compounds (**11a, 11b** and **11d**) was 23–26%.

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COMMUNICATION

Release of Lipoprotein Lipase from Cardiac Myocytes by Low-Molecular Weight Heparin

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Incubation of cardiac myocytes from rat heart with low-molecular weight heparin (LMWH; Mr approx. 3 kDa) for 30 min resulted in a concentration-dependent release of lipoprotein lipase (LPL) activity into the incubation medium. The release of lipoprotein lipase from cardiac myocytes isolated from both control and diabetic rat hearts induced by LMWH (10 μg/mL) following incubation times of 10 or 30 min was significantly greater than that produced by unfractionated heparin (10 and 30 μg/mL) or decavanadate (1 mM). Since LMWH released more LPL activity into the incubation medium than unfractionated heparin following a short (10 min) incubation time, LMWH is probably more effective in displacing LPL bound to heparan sulfate proteoglycan binding sites on the cell surface of cardiac myocytes.
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Lipoprotein lipase (LPL) catalyzes the hydrolysis of the triacylglycerol component of circulating lipoproteins at the luminal surface of the vascular endothelium (1). The enzyme is bound *via* electrostatic interactions to heparan sulfate proteoglycans on the surface of vascular endothelial cells (2,3). The *in vivo* administration of the anticoagulant heparin displaces LPL from its endothelial binding sites into the circulation as a lipase-heparin complex (post-heparin lipolytic activity). In the heart, LPL is synthesized in cardiac myocytes and then translocated to functional sites at the endothelium (4,5). LPL can be released into the medium of cardiac myocytes by short-term incubations with heparin (6,7), suggesting that the enzyme is also bound to heparan sulfate proteoglycans on the surface of parenchymal cells (8). The glycosaminoglycan specificity for the release of LPL from cardiac myocytes (6) was the same as observed for cultured endothelial cells (2).

Conventional (unfractionated) heparin is a heterogeneous polymer of repeating disaccharide sequences, with molecular weights ranging from 3–30 kDa (9). Low-molecular weight heparin (LMWH) subfractions are effective anticoagulants with improved pharmacokinetic properties and a reduced incidence of side effects (9). The intravenous administration of LMWH to rats or humans resulted in reduced plasma LPL activity compared to the administration of unfractionated heparin (10,11). Liu *et al.* (11) have recently demonstrated that LMWH was as effective as unfractionated heparin in releasing LPL from the capillary endothelium of perfused rat hearts and concluded that the lower plasma LPL activity measured after *in vivo* LMWH was due to the reduced ability of LMWH to prevent the rapid uptake of circulating LPL by the liver as compared to unfractionated heparin. The objective of our investigation was to compare the ability of LMWH and unfractionated heparin to displace LPL from the surface of cardiac myocytes.

MATERIALS AND METHODS

Preparation of cardiac myocytes. Calcium-tolerant cardiac myocytes were isolated from the hearts of male Sprague-Dawley (Charles River Canada Inc., St. Constant, Quebec, Canada) rats (200–300 g), essentially as described previously (12,13). The freshly isolated myocytes were resuspended in Joklik minimal essential medium supplemented with 1.2 mM $MgSO_4$ 1 mM carnitine, 1.5 mM $CaCl_2$ and 1% (wt/vol) defatted albumin (13) to a cell density of 4×10^5 cells/mL and were incubated for 10 or 30 min at 37°C with the indicated additions. The myocyte suspension was then centrifuged (10 s at 10,000 × *g* in a microcentrifuge), and the supernatant (medium) was removed for LPL assays (6). In some experiments, cardiac myocytes were isolated from rat hearts 3–4 d after the induction of diabetes by 100 mg/kg streptozotocin (13).

LPL assay. The activity of LPL in the post-heparin incubation medium was assayed with a sonicated [^{3}H]triolein (glycerol-[9,10-^{3}H]trioleate) substrate emulsion (12). The standard assay conditions were: 0.6 mM [^{3}H]triolein (1 mCi/mmol), 25 mM piperazine-N,N′-*bis*(2-ethanesulfonic acid) (PIPES), pH 7.5, 0.05% (wt/vol) defatted albumin, 50 mM $MgCl_2$, 2% (vol/vol) chicken serum as the LPL activator and 100 μL of post-heparin medium (13). After a 30-min incubation at 30°C, the release of [^{3}H]oleic acid was determined by liquid-liquid partitioning (12). All assays were performed in duplicate; LPL activity is routinely expressed as nmol oleate released per hour per 10^6 cells. LPL activity was linear with respect to the volume of post-heparin medium added to the assay, indicating that the introduction of different amounts of heparin into the assay had no effect on the determination of LPL activity.

Materials. Heparin (porcine intestinal mucosa) and LMWH (bovine intestinal mucosa; average molecular weight approx. 3 kDa) were obtained from Sigma Chemical Co. (St. Louis, MO) and were dissolved in water. Heparin was also obtained in solution (hepalean; 1000 U/mL) from Organon Teknika (Toronto, Ontario, Canada) (1 U/mL = 6 μg/mL). A 100 mM stock of decavanadate ($V_{10}O_{28}^{6-}$; yellow solution) was prepared by dissolving sodium orthovanadate (Na_3VO_4) in water and by adjusting the pH to 7.0 with concentrated HCl (14). [^{3}H]Triolein was purchased from Amersham Canada (Oakville, Ontario, Canada).

RESULTS AND DISCUSSION

Heparin produced a concentration-dependent increase in the release of LPL into the incubation medium of cardiac myocytes (Fig. 1). At every concentration (0.1–10 μg/mL) of

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Abbreviations: DV, decavanadate; LMWH, low-molecular weight heparin; LPL, lipoprotein lipase; PIPES, piperazine-*N,N*′-bis(2-ethanesulfonic acid).

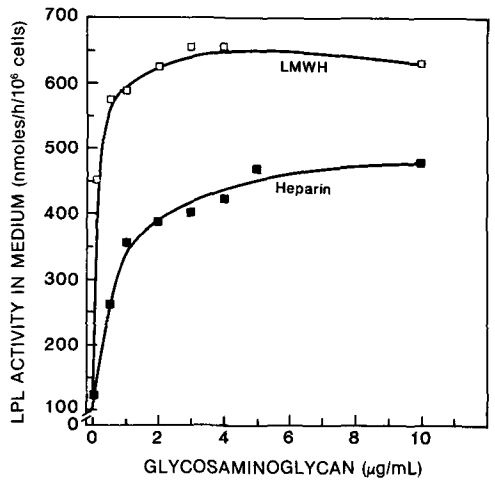

FIG. 1. Effect of heparin and low-molecular weight heparin (LMWH) concentrations on lipoprotein lipase (LPL) release from cardiac myocytes. LPL activity was determined in the incubation medium after cardiac myocytes were incubated for 30 min in the presence of the indicated concentrations of heparin (■) and LMWH (□).

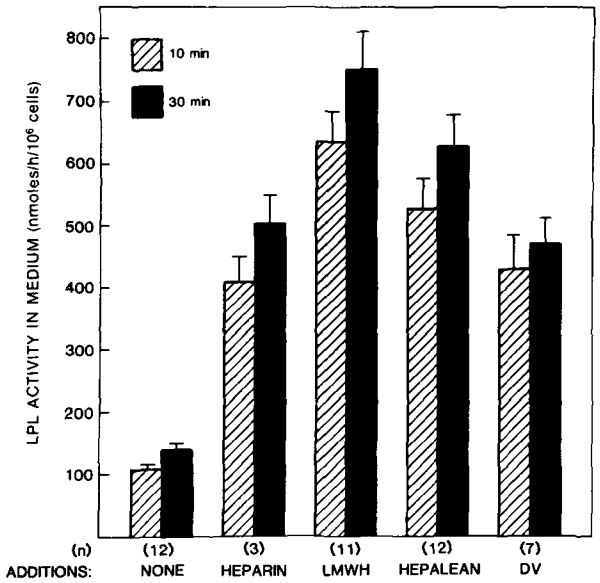

FIG. 2. Effect of heparin, low-molecular weight heparin (LMWH), and decavanadate (DV) on lipoprotein lipase (LPL) release from cardiac myocytes isolated from control rat hearts. Cardiac myocytes were incubated for 10 min (hatched bars) and 30 min (solid bars) with the following additions: heparin (10 μg/mL), heparin (hepalean; 5 U/mL or 30 μg/mL), LMWH (10 μg/mL) and DV (1 mM). LPL activity in the medium is presented as the mean ± SE for the number of experiments indicated in parentheses.

the glycosaminoglycans, incubation of cardiac myocytes with LMWH resulted in greater medium LPL activity than was measured after incubations with unfractionated heparin. Maximal LPL activity in the medium was observed at 3 μg/mL LMWH and 10 μg/mL heparin, suggesting that LMWH was more potent as well as being more effective in promoting the release of LPL into the medium of cardiac myocytes. On the other hand, if molar concentrations are calculated assuming Mr values of 3,000 and 12,000 (mean) for LMWH and heparin, respectively, then the optimal concentrations for LPL release (1.0 and 0.83 μM) were very similar. Interestingly, Liu *et al.* (15) have recently reported that a LMWH fraction (Mr 2,400–3,000) produced by enzymatic digestion was only one-tenth as potent by weight as a larger heparin fraction (Mr 12,700) in terms of release of LPL into plasma after intravenous injection into rats. Therefore, LMWH is much less effective (by weight) in displacing LPL from endothelial binding sites *in vivo* compared to the release of LPL from cardiac myocytes.

The heparin-induced release of LPL into the incubation medium of cardiac myocytes occurs in two phases: a rapid phase (5 to 10 min) followed by a slow phase (10 to 60 min) that is dependent on protein synthesis (6) and microtubular function (16). The rapid heparin-induced release of LPL was not decreased by cycloheximide or by a reduction in the incubation temperature from 37 to 23°C (6,16) and thus presumably reflects displacement of preformed enzyme from heparan sulfate proteoglycan binding sites on the myocardial cell surface (1,8,14). Incubation of cardiac myocytes with 10 μg/mL LMWH for 10 min (Fig. 2) resulted in a substantial release of LPL activity into the incubation medium (635 ± 52 nmol/h/10^6 cells) which was much greater than after incubation with unfractionated heparin (409 ± 41 nmoles/h/10^6 cells). Decavanadate (DV), a negatively charged polymerized vanadium compound, has recently been shown to mimic heparin and displace LPL from the surface of cardiac myocytes (14). As shown in Figure 2, medium LPL activity following incubations with 1 mM DV for 10 and 30 min was very similar to heparin-releasable LPL activity and thus substantially less than the LMWH-induced release of LPL.

Incubation of cardiac myocytes with a commercially available unfractionated heparin solution (hepalean; 5 U/mL or 30 μg/mL) resulted in slightly greater medium LPL activity than enzyme activity measured after incubation with the heparin solution prepared in our laboratory from the purchased powder (Fig. 2). Nevertheless, the net increment in medium LPL activity after incubation of control cardiac myocytes for 30 min with 10 μg/mL LMWH was 32 ± 4% greater ($P < 0.001$) than the heparin (hepalean)-induced release of LPL (Table 1). Braun and Severson (13) have reported that the heparin-induced release of LPL is reduced when cardiac myocytes are isolated from diabetic rat hearts. Incubation of diabetic cardiac myocytes with either heparin (hepalean) or LMWH resulted in lower LPL activity in the medium (Table 1) as compared to control myocytes, but the LMWH-induced release of LPL activity was still significantly greater (36 ± 7% increase) than the increment in medium LPL activity due to unfractionated heparin.

It is clear, therefore, that LMWH is significantly more effective than unfractionated heparin in displacing LPL from binding sites on the surface of cardiac myocytes; it follows that unfractionated heparin must not release the entire pool of LPL on the cell surface of cardiac myocytes. The observation by Rodrigues *et al.* (17) that preincubation of cardiac myocytes with unfractionated heparin reduced, but did not eliminate, the degradation of exogenous triolein in a subsequent incubation which is catalyzed by LPL bound to the surface of the myocardial cells (7) is consistent with this suggestion that the displace-

TABLE 1

Heparin- and Low-Molecular Weight Heparin (LMWH)-Induced Release of Lipoprotein Lipase (LPL) Activity into the Medium of Control and Diabetic Cardiac Myocytes[a]

Myocyte preparation	Net increment in medium LPL activity (nmoles/h/10^6 cells)		
	Heparin	LMWH	(%)[b]
Control (11)	470 ± 42	616 ± 50[c]	(32 ± 4%)
Diabetic (3)	325 ± 47	447 ± 84[c]	(36 ± 7%)

[a]Cardiac myocytes from either control or diabetic rat hearts were incubated for 30 min with no additions, 30 μg/mL heparin (5 U/mL hepalean), or 10 μg/mL LMWH, and the net increment of LPL activity in the incubation medium was measured (mean ± SEM) for the number of myocyte preparations indicated in parentheses.
[b]Percentage increase in the increment in medium LPL activity after LMWH compared to the heparin incubation.
[c]$P < 0.001$ (paired Student's t-test) relative to heparin.

ment of surface-bound LPL by unfractionated heparin is incomplete. The reduced mass of LMWH may permit a more effective interaction with endogenous heparan sulfate proteoglycans that are integrated into the glycocalyx on the plasma membrane so that the displacement of bound LPL is enhanced, although other factors must also play a role because LMWH was also more effective in releasing LPL than polymerized decavanadate which has an even lower molecular mass. LMWH may also complex differently with the dimeric LPL molecule (18). It must be acknowledged that unfractionated heparin and LMWH were isolated from porcine and bovine intestinal mucosa, respectively. Therefore, structural differences between two species, such as the degree of sulfation, may also contribute to the observed differences in promoting LPL release from cardiac myocytes. Previous experiments have shown that LPL activity released into the medium of cardiac myocytes was stable during incubation times up to 60 min (6). Therefore, the greater LPL activity in the medium after incubation with LMWH reflects enhanced release, not improved stability, of LPL in the medium of cardiac myocytes.

The increased effectiveness of LMWH to release LPL from isolated cardiac myocytes can be contrasted with results using other cell and tissue preparations. Unfractionated heparin and LMWH were not different with respect to their ability to release LPL from isolated adipocytes (10), whereas LMWH was slightly more effective than conventional heparin in promoting the release of LPL from the capillary endothelium of perfused hearts (11). These differences between the effectiveness of LMWH relative to unfractionated heparin will likely be a reflection of differences in the precise nature of the heparan sulfate proteoglycan binding sites for LPL on different cells. For example, LPL is bound to heparan sulfate proteoglycans that are covalently linked to the cell surface by a phosphatidylinositol-glycan membrane anchor in cardiac myocytes (13). In contrast, a similar pool of phosphatidylinositol-glycan-anchored heparan sulfate proteoglycans could not be detected on the surface of cultured aortic endothelial cells (3). Recently, Hoogewerf *et al.* (19) reported that the degree of sulfation of heparan sulfate proteoglycans influences the binding of LPL; cell-specific differences in the chemical composition of heparan sulfate proteoglycans could thus account for the observed variability in whether LMWH is more effective than unfractionated heparin in promoting the release of LPL from the surface of cells. Our results with cardiac myocytes reinforce the conclusion of Liu *et al.* (11) that lower plasma LPL activity following *in vivo* administration of LMWH is not due to a diminished ability to displace the enzyme from cellular binding sites, but rather is a consequence of more rapid hepatic clearance of circulating LPL released by LMWH as compared to unfractionated heparin.

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Effect of Simvastatin on Desaturase Activities in Liver from Lean and Obese Zucker Rats

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The effect of simvastatin, a hypocholesterolemic drug, on the biosynthesis of arachidonic acid was studied in obese and lean Zucker rats. After administration of 2 mg/kg body weight/d for 13 d, Δ6 and Δ5 desaturase activities were measured in liver microsomes at two substrate concentrations. In untreated rats, the Δ6 desaturation rate was similar in the obese and lean rats when measured at saturating substrate levels, whereas Δ5 desaturation was lower in the obese animals. Treatment with simvastatin did not change Δ6 desaturation in either phenotype but increased Δ5 desaturation in obese rats to reach the unchanged rate observed in lean animals. The changes were not reflected in the fatty acid composition of liver microsomal phospholipids when expressed as μg fatty acid/g of liver.
Lipids 28, 63-65 (1993).

Drugs such as resins or fibrates are commonly prescribed as therapeutic agents for the treatment of hypercholesterolemia. Simvastatin, also a hypocholesterolemic agent, belongs to a new series of competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (CoA) reductase, the key regulatory enzyme of mammalian cholesterol biosynthesis (1-5). A recent study showed changes in fatty acid profiles of hepatic microsomal phospholipids from simvastatin treated Wistar rats, which suggested an effect of the drug on the activity of one or several desaturases involved in the biosynthesis of n-6 polyunsaturated fatty acids (6).

The genetic obesity of Zucker rats is often associated with hyperlipidemia. Increased rates of lipogenesis (7,8) and Δ9 desaturase activity (9) with concomitantly decreased activity of fatty acid (FA) oxidation (10) in the liver were the major abnormalities of the obese (fa/fa) animal as compared to its littermate (Fa/-). There was also an inhibition of linoleic acid (18:2n-6) conversion into arachidonic acid (20:4n-6), resulting from decreased Δ5 desaturation activity (11).

The purpose of the present study was to compare obese with lean Zucker rats with respect to the effect of simvastatin on the *in vitro* activities of hepatic Δ6 and Δ5 desaturases. The fatty acid composition of microsomal phospholipids was also studied in order to examine whether simvastatin treatment induced changes in the biosynthesis of n-6 and n-3 polyunsaturated fatty acids *in vivo*.

MATERIALS AND METHODS

[1-^{14}C]Linoleic acid and [2-^{14}C]dihomo-γ-linolenic acid (56 mCi/mmol, 97% radiochemical purity) were purchased from the Radiochemical Centre (Amersham, U.K.). The substrates were diluted with the corresponding unlabelled fatty acid using ethanol as solvent. Cofactors and biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Male obese rats (fa/fa) of the Zucker strain were purchased from INSERM U177 (Paris, France). They were 12 weeks old on arrival at the laboratory and were given free access to a standard laboratory chow (A04 from UAR, Villemoisson sur Orge, France) and tap water. The animals were divided into four groups of six animals each; two groups of untreated (control), both obese and lean, and two groups of treated animals. From days 1 to 13 of the experiment, treated rats received a daily dose of 2 mg/kg body weight of simvastatin in 0.1 mL of corn oil by gastric intubation. For each phenotype, control rats received only the equivalent volume of excipient. On day 13, animals were treated as above, but they were kept fasting 16 h up to killing by decapitation on day 14. The liver of each rat was removed, weighed and perfused with ice-cold 0.154 M KCl solution and then homogenized in 5 vol of sucrose and 0.05 M phosphate (pH 7.4) solution using a Teflon Potter Elvejhelm homogenizer. The preparation of microsomal fractions from *ca.* 3 g of liver was done as described previously (11). The protein content of microsomal fractions was determined by the Biuret method (12).

Five mg microsomal protein was incubated in an open flask with [1-^{14}C]18:2n-6 or [2-^{14}C]20:3n-6. The substrate levels (60 nmol and 30 nmol, respectively) were nonsaturating so they would be close to physiological concentration. In addition, saturating levels, 120 and 60 nmol respectively, also were used to evaluate the Δ6 and Δ5 desaturation capacity of liver microsomes. Incubations were performed at 37°C in a shaking water bath for 15 min with a total volume of 2.1 mL incubation medium containing in mmol/L: phosphate buffer (pH 7.4) 72, $MgCl_2$ 4.8, CoA 0.5, adenosine 5′-triphosphate 3.6, and reduced nicotinamide adenine dinucleotide phosphate 1.2. Incubations were stopped by adding 15 mL of chloroform/methanol (1:1, vol/vol). The lipids were saponified, and the extracted fatty acids were methylated with methanol/boron trifluoride (13). The conversion of labelled substrates (linoleic and dihomo-γ-linolenic acids, respectively) into their Δ6 n-6 and Δ5 n-6 products (γ-linolenic and arachidonic acids, respectively) was determined after separation by reversed phase high-performance liquid chromatography as described by Narce *et al.* (14). Fractions corresponding to individual FA methyl ester were collected at the detector outlet (differential refractometer), and radioactivity was measured in the sample by liquid scintillation counting.

Lipids from aliquots of liver microsomes were extracted with dimethoxymethane/methanol (4:1, vol/vol) according to Delsal (15). Phospholipids were separated by silicic acid column chromatography (16). After heptadecanoic acid was added as internal standard, FA were transmethylated (13). Methyl esters were analyzed by gas-liquid chromatography using a Becker Packard Model 419 gas chromatograph (Packard Instruments, Rungis, France) equipped with a laboratory-made 30 m × 0.4 mm i.d. glass capillary

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Abbreviations: CoA, coenzyme A; FA, fatty acid.

column coated with Carbowax 20 M (Applied Science, State College, PA). Peaks on the chromatograms were assigned based on their retention time relative to methyl heptadecanoate. Peak areas were measured using a Delsi Enica 21 integrator (Delsi Instruments, Suresnes, France).

Data presented are means ± SD. After analysis of variance using a Duncan's multiple range test, means were compared according to the least significant difference ($P < 0.01$) and classified in increasing order.

RESULTS AND DISCUSSION

In the untreated obese rats, the Δ6 desaturase activity at the saturating level of substrate was not significantly different from that observed in lean rats (Table 1). However, at half-saturating concentration, the Δ6 desaturation rate was higher in obese rats, contrary to what was observed previously in unfasted animals (11). The Δ6 desaturase affinity for the substrate might be slightly higher in the fasted obese rat, a condition which would not appear in the presence of a high level of substrate. In unfasted rats, the presence in microsomes of free unlabelled dietary linoleic acid from the blood, which are probably present in higher amounts in the hyperphagic obese rats, may decrease the apparent Δ6 desaturation rate in obese rats. The treatment with simvastatin did not induce any change in Δ6 desaturation in either phenotype or at either substrate concentration. On the contrary, the Δ5 desaturase activity was lower in untreated obese rats as compared to lean rats at both substrate concentrations (Table 1). This was probably due to a lower affinity of the enzyme for the substrate, as shown previously (11). Treatment by simvastatin restored the desaturation rate to the control level in obese rats, but had no significant effect on the lean animals. The same effect had been observed with fenofibrate, another hypolipidemic drug (17).

In liver microsomal phospholipids (Table 2) the amount of total FA per gram of liver was lower in untreated obese rats than in lean rats, contrary to what was previously observed in total microsomal liver lipids of 12-week-old rats (11). In obese rats, simvastatin treatment decreased the amount of 18:2n-6, 20:3n-6 and of total n-6 fatty acids, but not 20:4n-6. This effect was not observed in lean rats. The total n-6 plus n-3 FA from microsomal phospholipids

TABLE 1

Δ6 and Δ5 Desaturase Activities in Liver Microsomes from Lean and Obese Zucker Rats[a]

		Δ6 Desaturation 18:2→18:3n-6		Δ5 Desaturation 20:3→20:4n-6	
Phenotype	Treatment	60[b]	120[c]	30[b]	80[c]
Lean	Control	1.8 ± 0.3[d]	3.0 ± 0.5[d]	10.6 ± 0.6[e]	15.8 ± 1.3[e]
	Treated	2.0 ± 0.2[d]	3.7 ± 0.5[d]	11.2 ± 0.5[e]	15.2 ± 1.4[e]
Obese	Control	2.6 ± 0.2[e]	3.6 ± 0.2[d]	8.7 ± 0.5[d]	11.8 ± 1.9[d]
	Treated	2.5 ± 0.2[e]	3.5 ± 0.3[d]	10.6 ± 0.8[e]	15.1 ± 1.8[e]

[a] Desaturase activities are expressed as nmoles of substrate converted per 15 min per 5 mg microsomal protein. Results are means ± SD of six animals in each group. Means were compared in each column and classified according to increasing order. Means assigned different superscript letters were significantly different ($P < 0.01$).

[b] Nonsaturating substrate concentration (nmoles).

[c] Saturating substrate concentration (nmoles).

TABLE 2

Concentration (μg/g of liver) of Total and Polyunsaturated Fatty Acids in Liver Microsomal Phospholipids from Lean and Obese Zucker Rats[a]

	Phenotype			
	Lean		Obese	
Fatty acids	Control	Treated	Control	Treated
Total	2510 ± 400[e]	2500 ± 200[e]	1530 ± 100[d]	1300 ± 260[d]
18:2n-6	283 ± 30[f]	275 ± 10[f]	143 ± 14[e]	111 ± 12[d]
20:3n-6	19 ± 7[d,e]	16 ± 4[d]	37 ± 5[f]	25 ± 4[e]
20:4n-6	695 ± 55[e]	700 ± 20[e]	330 ± 11[d]	298 ± 20[d]
22:6n-3	258 ± 12[e]	270 ± 12[e]	226 ± 12[d]	200 ± 13[d]
Total n-6[b]	1013 ± 53[f]	1013 ± 13[f]	520 ± 11[e]	442 ± 9[d]
Total n-3[c]	310 ± 14[f]	315 ± 15[f]	283 ± 12[e]	240 ± 9[d]
n-6 plus n-3	1323 ± 50[f]	1328 ± 14[f]	803 ± 12[e]	682 ± 9[d]

[a] Results expressed as μg fatty acid/g of liver are means ± SD for n = 6 animals in each group. Within each line, means not showing a common superscript letter are significantly different ($P < 0.01$). They are classified according to increasing order.

[b] Total n-6 includes 18:3, 20:2, 22:4 and 22:5n-6, not listed individually.

[c] Total n-3 includes 18:3, 20:5 and 22:5n-3, not listed individually.

was 39% lower in untreated obese rats than in untreated lean animals. However, when expressed as a percentage of total fatty acids, there was no change, and the percentage (52.5% in both cases) was similar to that found in treated animals.

The mechanism by which simvastatin increases the Δ5 desaturation rate is unknown. Fibrates have been shown to exhibit the same effect (17,18). They are also proliferators of peroxisomes (19), and some desaturation activities have been observed in these organelles (20). This may explain the increased rate of Δ5 desaturation in fenofibrate treated obese rats (17). Because simvastatin is not a peroxisome proliferator (6), this mechanism cannot be evoked here. Alternatively, changes in the hormonal status of simvastatin-treated obese rats cannot be ignored as several hormones, such as insulin, increase the Δ5 desaturation rate (21). However, further studies are needed to test this hypothesis.

The increase in Δ5 desaturation rate in treated obese rats was not accompanied by an increase in the amount of arachidonic acid in liver microsomal phospholipids. Because of the relatively low turnover rate of these constituents, prolonged drug treatment may be necessary to restore a fatty acid profile in obese rats similar to that observed in lean rats.

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Composition of the Phospholipids and Their Fatty Acids in the ROC-1 Oligodendroglial Cell Line

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ROC-1 cells are a hybrid of C-6 rat glioma and rat oligodendroglia cells. Biochemically these cells resemble the oligodendroglia parent, but their lipid composition is unknown. The phospholipid composition in mole % was: cardiolipin, 1.0; phosphatidylglycerol, 1.2; ethanolamine glycerophospholipids, 27.6; phosphatidylinositol, 5.8; lysophosphatidylethanolamine, 0.8; phosphatidylserine, 5.6; choline glycerophospholipids, 43.7; sphingomyelin, 13.7; phosphatidylinositol-4-monophosphate, 0.8; and lysophosphatidylcholine, 0.6. The choline and ethanolamine plasmalogens made up 7.2 and 18.4% of the total phospholipids, respectively. The phospholipid composition reflects that of both parental cells. The cells had moderate to high levels of 20:3n-9 indicating n-6 series fatty acid deficiency. The phosphatidylinositol had very high 20:3n-9 levels with a 20:3n-9/20:4n-6 ratio of 2.1 compared to 0.44 and 0.58 for ethanolamine glycerophospholipids (EtnGpl) and choline glycerophospholipids (ChoGpl) respectively. The saturated/polyenoic fatty acid ratios were 0.40 for EtnGpl, 3.38 for ChoGpl and 1.48 for phosphatidylinositol.
Lipids 28, 67-71 (1993).

Phospholipids and their constituent fatty acids in the cell membrane have a major role in maintaining membrane structure, integrity and function including receptor-mediated signal transmission (1-8). Many different cell lines are used to study these areas, but for many of the cells the phospholipid and fatty acid compositions are unknown.

The partial phospholipid composition of oligodendroglia has been reported (9-12) as well as the composition of C-6 glioma cells (13). The fatty acid composition of oligodendroglia (14) and the fatty acid composition of oligodendroglia polar lipids (11) have also been reported.

In this communication, we report the phospholipid and fatty acid composition of ROC-1 cells. ROC-1 oligondendroglia cells are a hybrid between a C-6 rat glioma and a rat oligondendrocyte (15).

MATERIALS AND METHODS

ROC-1 cells were obtained from F.A. McMorris (Wistar Institute of Anatomy and Biology, Philadelphia, PA). The cells were maintained in modified Eagle's medium supplemented with 10% fetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine. The cells were fed by removing half of the culture medium and replacing it with fresh culture medium. The cells were maintained in a 95% humidified air, 5% CO_2 atmosphere at a constant temperature of 37°C. Upon reaching confluency, medium was removed and cells were washed with phosphate-buffered saline (PBS) to remove any detached cells. Trypsin was added to remove attached cells and these cells were then suspended in medium. The cell suspensions were split and plated upon 100-mm polystyrene culture plates.

Confluent cells were extracted using *n*-hexane/2-propanol (3:2, vol/vol) (16). Compared with chloroform/methanol, this extraction method gives better recovery of lipids with less protein contamination. Prior to extraction, the medium was removed and the cells were washed with two 3-mL portions of cold PBS buffer to remove traces of medium. The plates were immediately placed upon dry ice and frozen to minimize acylhydrolase activity during extraction (17). Two 2-mL aliquots of 2-propanol were used to remove the cells from the cultured dishes. The initial aliquot was added to the frozen cells which were subsequently removed from the plate using a Teflon cell scraper. The second aliquot was used to rinse the plate. Both of these aliquots were added to a tube containing 6-mL of *n*-hexane for a final *n*-hexane/2-propanol ratio of 3:2 by volume. The extracts were kept under nitrogen to minimize autoxidation of lipids.

Prior to phospholipid separation. lipid extracts were ultrafiltered using a Rainin 0.2 μm nylon filter (Woburn, MA). The sample was dried under a stream of nitrogen and redissolved in a known volume of *n*-hexane/2-propanol/water (56.7:37.8:5.5, vol/vol/vol) before high-performance liquid chromatography (HPLC).

The cell lipid extracts were separated into major phospholipid classes by HPLC including the separation of the acidic phospholipids phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns), as well as lysophosphatidylethanolamine (lysoPtdEtn) (18). The lower limit of sensitivity was 100 nmol of injected phospholipid. Each phospholipid class was quantitated by measuring lipid phosphorus (19). The solvents, HPLC grade *n*-hexane and 2-propanol from E.M. Science (Cherry Hill, NJ), were filtered through a 0.5 μm Millipore FH-type nylon filter (Bedford, MA) and degassed. Solvent A was *n*-hexane/2-propanol (3:2, vol/vol) and solvent B was *n*-hexane/2-propanol/water (56.7:37.8:5.5, vol/vol/vol). Water was purified using a Millipore water purification system. The HPLC consisted of two Altex (Berkeley, CA) 100A pumps, an Altex 420/421 controller and an Altex model 210 injection port. The Dupont (Wilmington, DE) Zorbax Silica column (4.6 mm × 250 mm, 5-6 μm) was maintained at a constant temperature of 34°C with a Jones Chromatography (Lakewood, CO) heating block. An ISCO (Lincoln, NE) V4 ultraviolet (UV) variable wavelength detector was used to monitor absorption at 205 nm.

Plasmalogens were assayed by HPLC after mild acidic hydrolysis of the previously collected ethanolamine glycerophospholipid (EtnGpl) and choline glycerophospholipid (ChoGpl) fractions (20). This caused hydrolysis of the

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Abbreviations: CerPCho, sphingomyelin; ChoGpl, choline glycerophospholipids; EtnGpl, ethanolamine glycerophospholipids; FA, fatty acid; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; Gpl, glycerophospholipid; HPLC, high-performance liquid chromatography; lysPtdEtn, lysophosphatidylethanolamine; PBS, phosphate-buffered saline; PlsCho, plasmenylcholine; PlsEtn, plasmenylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PUFA, polyunsaturated fatty acid; RCF, relative correction factors; TLC, thin-layer chromatography; UV, ultraviolet.

alkenyl ether bond of the plasmalogens while the alkylacyl and diacyl glycerophospholipids (Gpl) remained intact. The sample was reextracted and rechromatographed utilizing the same separation procedure as used in the initial separation. The lysophospholipid fractions were collected for phosphorus assay (19). The fraction containing the alkylacyl and diacyl Gpl was then collected and subjected to alkaline transmethylation in 2 mL of 0.1 M NaOH in methanol. After 15 min the reaction was terminated with addition of 1-mL ethyl formate (21). The two reaction products were separated by the addition of chloroform/isobutanol/water (4:2:3, vol/vol/vol) to the methanol followed by centrifugation to complete the phase separation. The top layer contained glycerophosphocholine or glycerophosphoethanolamine and the bottom layer contained the lysophospholipid. Aliquots were taken for phosphorus assay (19).

Phospholipid acyl chains were converted to fatty acid methyl esters (FAME) through base-catalyzed transesterification (22). This method, unlike BF_3-catalyzed transesterification, avoids oxidative side reactions and the formation of dimethylacetals from plasmalogens. FAME were extracted with 2-mL of hexane. The upper phase was pipetted off and the remaining methanol phase washed with two 2-mL aliquots of hexane. FAME were stored in a nitrogen atmosphere at −20°C.

FAME were separated and quantitated by gas-liquid chromatography (GLC). A set of standards (NuChek Prep, Elysian, MN) was used to establish relative retention times and relative correction factors (RCF) for each FAME. The internal standard was 17:0 methyl ester. The detector response was linear within the concentration range of the samples for all identified fatty acids of varying chain lengths and degrees of unsaturation with correlation coefficients of 0.985 or greater. This standard was used to establish standard curves and for each sample run.

The GLC system was comprised of a Shimadzu (Kyoto, Japan) GC-8A, a Supelco (Bellefonte, PA) SP-2330 capillary column (30 m long) and a flame-ionization detector. Column temperature was maintained at 190°C with nitrogen as the carrier gas at a pressure of 0.5 kg/cm^2. Detector and injector temperatures were maintained at 220°C. A split ratio of 100:1 was used. Peak area data were collected with a Nelson Analytical 760 series intelligent interface (Cupertino, CA) and computed with Nelson model 2600 software.

RESULTS

The ChoGpl comprise the largest proportion of the phospholipids in ROC-1 cells (Table 1). The order of the other phospholipids in decreasing proportions is EtnGpl > sphingomyelin (Cer*P*Cho) > PtdIns = PtdSer. The lysophospholipids make up a small portion of the total phospholipid.

The compositions of EtnGpl and ChoGpl subclasses are reported in Table 2. Ethanolamine plasmalogen (PlsEtn), the alkenylacyl subclass, accounted for 18.4 mol% of the total phospholipids. Of the ChoGpl subclasses, the diacyl subclass was the largest fraction making up 33.0 mol% of the total phospholipids. There was a large amount of choline plasmalogen (PlsCho), accounting for 7.2% of the phospholipids.

The fatty acid (FA) compositions of EtnGpl, ChoGpl and PtdIns are reported in Table 3. For EtnGpl the composition of the major FA in decreasing mole percent was: 20:4n-6 > 18:1n-9 > 18:0 > 20:3n-9 > 22:6n-3. There was a relatively large amount of the Mead acid, 20:3n-9. The 20:3n-9/20:4n-6 ratio was 0.43. For ChoGpl, the composition of the major FA in decreasing mole percent was: 18:1n-9 > 16:0 >> 18:0 > 20:4n-6. The 20:3n-9/20:4n-6 ratio was 0.58. The PtdIns FA composition in decreasing mole percent was: 18:0 > 18:1n-9 > 20:3n-9 > 16:0 >18:2n-6 = 20:4n-6. There was a large proportion of the Mead acid present, accounting for 12.6% of the total FA. The 20:3n-9/20:4n-6 ratio was 2.1.

The EtnGpl FA composition was the most unsaturated, with a saturated/unsaturated fatty acid ratio of 0.27. The unsaturation index for ChoGpl and PtdIns was 0.64 and 0.69, respectively. The saturated/monoenoic fatty acid ratios for ChoGpl and EtnGpl were essentially the same; however, this ratio was higher for PtdIns. There were large differences in the saturated/polyenoic fatty acid ratios for the phospholipid classes. These ratios were 0.40 for EtnGpl, 3.38 for ChoGpl and 1.48 for PtdIns.

TABLE 1

Phospholipid Composition of ROC-1 Cells[a]

Phospholipid	mol %
Cardiolipin	1.0 ± 0.4
Phosphatidylglycerol	1.2 ± 0.7
Ethanolamine glycerophospholipids	27.6 ± 3.3
Phosphatidylinositol	5.8 ± 1.4
Lysophosphatidylethanolamine	0.8 ± 0.8
Phosphatidylserine	5.6 ± 1.5
Choline glycerophospholipids	43.7 ± 5.1
Sphingomyelin	13.7 ± 2.8
Phosphatidylinositol 4-phosphate	0.8 ± 0.2
Lysophosphatidylcholine	0.6 ± 0.2

[a]The values are means ± SD and are expressed as mole percent of each phospholipid class. For each value n=5.

TABLE 2

Composition of Ethanolamine and Choline Glycerophospholipid (Gpl) Subclasses[a]

Phospholipid subclasses	Gpl (%)	Total phospholipid (%)
Ethanolamine glycerophospholipid (EtnGpl)		
Diacyl	25.6 ± 1.5	7.1
Alkylacyl	7.6 ± 0.9	2.1
Alkenylacyl	66.8 ± 1.9	18.4
Choline glycerophospholipid (ChoGpl)		
Diacyl	75.5 ± 1.8	33.0
Alkylacyl	8.1 ± 1.3	3.5
Alkenylacyl	16.4 ± 1.7	7.2

[a]Values are means ± SD and are expressed as mole percent of the individual glycerophospholipid class. The total % phospholipid represents the mole percentage of each subclass with respect to total phospholipid composition. For EtnGpl n=5 and for ChoGpl n=7.

DISCUSSION

One inherent problem of growing primary oligodendroglial cells in culture is the difficulty of isolating cells from

TABLE 3

Fatty Acid Composition of Ethanolamine and Choline Glycerophospholipids, and Phosphatidylinositol[a]

Fatty acid	Ethanolamine glycerophospholipid (EtnGpl)	Choline glycerophospholipid (ChoGpl)	Phosphatidylinositol (PtdIns)
16:0	3.8 ± 1.3	32.7 ± 3.7	7.8
16:1n-9	0.8 ± 0.5	3.8 ± 1.5	3.0
18:0	17.5 ± 4.9	6.5 ± 1.1	32.8
18:1n-9	23.9 ± 2.2	43.6 ± 3.1	28.4
18:2n-6	1.8 ± 0.4	2.8 ± 2.1	5.6
18:3n-6	0.5 ± 0.1	0.2 ± 0.2	0.3
18:3n-3	0.7 ± 1.0	0.6 ± 0.5	0.8
20:1n-9	1.1 ± 0.4	0.7 ± 0.3	—
20:2n-6	0.8 ± 0.5	1.0 ± 0.8	—
20:3n-9	10.9 ± 0.8	1.8 ± 0.2	12.6
20:3n-6	0.4 ± 0.4	0.4 ± 0.1	—
20:4n-6	24.9 ± 1.5	3.1 ± 0.3	6.0
20:5n-3	2.9 ± 1.7	—	—
22:1n-9	0.6 ± 0.4	—	—
22:2n-6	0.7 ± 0.9	0.1 ± 0.1	—
22:3n-3	1.0 ± 0.3	0.9 ± 0.3	2.0
22:4n-6	1.9 ± 0.8	0.2 ± 0.2	—
22:6n-3	6.1 ± 1.0	0.5 ± 0.3	—
24:1n-9	—	0.8 ± 1.3	—
20:3n-9/20:4n-6 ratio	0.44	0.58	2.1
n-3/n-6 ratio	0.37	0.40	0.44
Monoenoic	26.4 ± 3.5	48.9 ± 6.2	31.4
Polyenoic	52.6 ± 9.4	11.6 ± 6.8	27.3
Unsaturated	79.0 ± 13.2	60.5 ± 13.0	58.7
Saturated	21.3 ± 6.2	39.2 ± 4.8	40.6
Saturated/unsaturated	0.27	0.64	0.69
Saturated/monoenoic	0.81	0.80	1.29
Saturated/polyenoic	0.40	3.38	1.48

[a]Values are mol% for each fatty acid for the respective phospholipid class. The values for PtdIns represent the means of two determinations. For ChoGpl and EtnGpl the values represent mean ± SD for 4 and 5 determinations, respectively.

tissue (23,24). Clonal cell lines are useful because large quantities can be grown with little or no difference between batches and no contamination from other cell types as commonly occur with primary cell cultures. The ROC-1 cell hybrid is the best oligodendroglia cell line now available and has morphological and biochemical characteristics that closely resemble those of the primary oligodendrocyte (25).

The ROC-1 oligodendroglial cell is a hybrid cell made from a rat oligodendrocyte and a C-6 rat glioma cell (15). Thus, the phospholipid composition should closely resemble one or both of the parent cell types. The Cer*P*Cho, EtnGpl, ChoGpl and cardiolipin mol% phospholipid composition of ROC-1 cells is similar to that of C-6 glioma cells (13). PtdIns and PtdSer levels reflect those in glial cells (9). These values are found in Table 4 for comparison. In the study using whole oligodendroglia, the PtdIns and PtdSer were not resolved from each other and the two classes made up 10.4% ± 0.6 of the total phospholipid composition (10). Our values for the phospholipid composition of ROC-1 cells agree with these reported values. Thus, the ROC-1 cell phospholipid composition is a hybrid, with a composition similar to the composition of both parent cells.

The ROC-1 cells had a lower ChoGpl content (43.7%) than reported for fresh oligodendroglia preparations 50.6% ± 4.8 (10) and 49.6% (12). Our lower value may reflect the complete separation of Cer*P*Cho from the ChoGpl which we achieve using HPLC. The EtnGpl composition (27.6%) was slightly higher than fresh oligodendroglia preparations 23.0% ± 2.6 (10) and 23.6% (12). Our values for PtdIns and PtdSer agree with those reported by other workers (10,12), suggesting the PtdIns and PtdSer composition closely resembled the oligodendroglial parent cell.

We report compositional values for the EtnGpl and ChoGpl subclasses. In freshly isolated glial cells from 14-day-old rat, the ethanolamine plasmalogen made up 7.5% of the total phospholipid compared to our value of 18.4%, but no choline plasmalogen was reported (9). ROC-1 cells contained a high proportion of choline plasmalogen (7.2% of the total phospholipid) and high proportions of 1-*O*-alkyl-2-acyl-glycerophosphoethanolamine and 1-*O*-alkyl-2-acyl-glycerophosphocholine. These two phospholipid classes made up 2.1 and 3.5% of the total phospholipids, respectively.

The fatty acid compositions of the EtnGpl, ChoGpl and PtdIns are reported in Table 3. The EtnGpl had a low saturated/unsaturated fatty acid ratio. This is partly due to the high ethanolamine plasmalogen proportion which contributes a large amount of 20:4n-6. There was a large proportion of 20:3n-9 (Mead acid) in the EtnGpl. Mead acid is thought to be increased in PUFA deficient cells in an attempt to maintain a normal saturated/unsatu-

TABLE 4

Comparison of Phospholipid Compositions of ROC-1, C-6 Glioma and Glial Cells[a]

Phospholipid	ROC-1 n=5	C-6 glioma[b] n=5	Mixed glial[c] n=9
Cardiolipin	1.0 ± 0.4	1.9 ± 0.7	2.5
Sphingomyelin	13.7 ± 2.8	8.1 ± 1.1	7.0
Ethanolamine glycerophospholipids	27.6 ± 3.3	28.5 ± 1.7	22.9
Choline glycerophospholipids	43.7 ± 5.1	52.2 ± 2.3	52.4
Phosphatidylinositol	5.8 ± 1.4	3.8 ± 0.5	5.8
Phosphatidylserine	5.6 ± 1.5	4.5 ± 0.5	6.2

[a]The values are mole percent of total phospholipid. ROC-1 phospholipids were separated by high-performance liquid chromatography and quantitated as lipid phosphorus. Values are means ± SD.
[b]The C-6 glioma cell phospholipids were separated by thin-layer chromatography (TLC) and quantitated as lipid phosphorus (13).
[c]The glial cell phospholipids were separated by two-dimensional TLC and quantitated as lipid phosphorus (9).

rated fatty acid index (26). The ChoGpl and PtdIns have even higher 20:3n-9/20:4n-6 ratios. This increase in 20:3n-9/20:4n-6 ratio indicates the apparent need to maintain a high level of PUFA in the cell through the increase in 20:3n-9 levels. Overall, the ROC-1 cells are deficient in the n-6 series, particularly in arachidonic acid as indicated by the higher 20:3n-9 proportions.

Cultured murine brain cells have a 20:3n-9/20:4n-6 ratio of 0.27 for cells grown in 10% fetal calf serum and 1.6 for cells grown in a chemically defined medium devoid of PUFA (14). We found a 20:3n-9/20:4n-6 ratio of 0.44 for EtnGpl, 0.58 for ChoGpl and 2.1 for PtdIns. These results indicate that the ROC-1 cells were in a state of PUFA deficiency. The two major phospholipid classes had moderate PUFA deficiency, while the PtdIns was severly PUFA deficient.

Oligodendrocytes in culture for 34 d have a saturated/polyenoic fatty acid ratio of 0.41 for fatty acids from polar lipids (11). Cells cultured for only 8 d have a saturated/polyenoic ratio in the total lipid extract of 1.29 for serum-supplemented cells, 1.86 for cells grown in a chemically defined medium deficient in PUFA and 0.84 for cells in culture for 34 d in a chemically defined medium supplemented with PUFA (26). Our cells were grown in medium supplemented with 10% fetal calf serum and were not supplemented with exogenously added PUFA. The saturated/monoenoic fatty acid ratio was essentially the same in EtnGpl and ChoGpl, *i.e.*, 0.81 and 0.80, respectively. The saturated/monoenoic ratio for PtdIns was 1.29. ChoGpl had a saturated/polyenoic fatty acid ratio of 3.38 compared to a ratio of 0.40 for EtnGpl and 1.48 for PtdIns. Clearly, the ChoGpl had a lower polyenoic fatty acid content, although this is generally the case for the more saturated ChoGpl. Regardless of the variation in the saturated/polyenoic fatty acid ratios, the values for ROC-1 cells indicate a decrease in PUFA compared to oligodendroglia cells grown under similar conditions. These differences cannot be explained due to medium conditions since the cells were grown in 10% fetal bovine serum. Further, the ROC-1 cells had a larger proportion of 20:3n-9 indicating that the cells were PUFA deficient, even though the culture conditions are known not to produce PUFA deficiency in other cells. The lower levels of PUFA in ROC-1 cells may be directly related to the tumor origin of the cell line. This deficiency may cause possible signal transduction problems and an interruption of normal cellular events.

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A Simple and Efficient Synthesis of Fatty Thioacids

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A rapid and high-yielding two-step synthesis of fatty thioacids from readily available starting materials has been devised. In the first step, an acid chloride is reacted with thioacetic acid at room temperature to produce a mixed thioanhydride, which in the second step is nucleophilically deacetylated with propylamine or butylamine at 5°C. Each step is complete in five minutes and proceeds in quantitative yield. The versatility of the procedure is demonstrated by the synthesis of fatty thioacids from six to sixteen carbons in length.
Lipids 28, 73-74 (1993).

Replacement of oxyester with thioester functions provides alternate lipolytic enzyme substrates that are close structural mimics of physiological lipid subtrates. Hydrolysis of thiolipid substrates can be followed in coupled spectroscopic assays and thus has analytical advantages over traditional radiochemical assays. For example, thiolipid assays for phospholipase A_1 (1,2) and phospholipase A_2 (3,4) have been described.

Hendrickson and colleagues (5,6) have devised synthetic methods for the preparation of racemic and enantiomeric thiophospholipid substrates of phospholipases A_1 and A_2. Their procedure uses $Na_2S{\cdot}9H_2O$ and the appropriate acid chloride to synthesize thioacids (6). However, the nonahydrate of Na_2S must be dehydrated, typically by drying *in vacuo* over P_2O_5 for two days, before reaction with the acid chloride, and yields are only ~50%. Residual water, or adventitious water contamination, promotes the formation of anhydrides and oxyacids as side products, and thus inert-atmosphere techniques are required to achieve maximal yields of thioacids. Moreover, the reaction mixture is a slurry because Na_2S is poorly soluble in the solvent, pyridine. An alternate method, reaction of H_2S with the appropriate acid chloride (7), has several disadvantages: extreme stench and toxicity of H_2S; a complicated experimental apparatus is required to contain H_2S; the reaction is conducted at −30°C, far from ambient temperature.

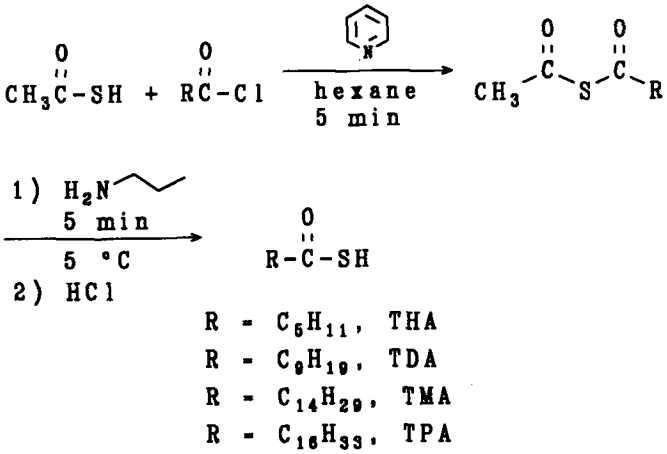
In this communication we describe a rapid, efficient and high-yielding synthesis of fatty thioacids that avoids these complications.

EXPERIMENTAL PROCEDURES

Materials. Thioacetic acid (TAA), hexanoyl chloride, decanoyl chloride, myristoyl chloride, palmitoyl chloride, propylamine (PA) and anhydrous pyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI). TAA was usually distilled before use. Butylamine (BA) was purchased from Matheson, Coleman and Bell (Norwood, OH). Concentrated HCl was purchased from Fisher Scientific (Fair Lawn, NJ). High-performance liquid chromatography (HPLC)-grade hexane was from EM Science (Cherry Hill, NJ). Flexible plastic thin-layer chromatography (TLC) plates (Polygram SIL G/UV_{254}, silica gel with fluorescent indicator) were purchased from Brinkman Instruments, Inc. (Lauda-Königshofen, Germany) and were produced by Macherey-Nagel (Düren, Germany).

Synthesis and product characterization. Scheme 1 outlines the procedure for the thioacid synthesis; the preparation of thioadecanoic acid (TDA) is illustrative of the methods used. Except where specified, all manipulations were performed at room temperature. Reaction mixtures were analyzed by TLC, with hexane/diethyl ether (7:3, vol/vol) as the mobile phase. In a round-bottom flask (RBF) equipped with a magnetic stirrer, 4.2 mL of decanoyl chloride (0.02 mol) and 1.5 mL of TAA; (0.021 mol) were dissolved in 100 mL of hexane that contained 2.0 mL (0.025 mol) of pyridine, and the reaction mixture was stirred for 5 min. TLC of the reaction mixture showed a new spot at R_f 0.7 for the mixed acetic-decanoic thioanhydride. The reaction mixture was then cooled in an ice bath, 3.4 mL of propylamine (0.041 mol) was added and stirring was continued for 5 min. Use of an ice bath was necessary to moderate the exothermic reaction of the amine with the mixed anhydride. After these reactions, the acid chloride and mixed thioanhydride spots were no longer detected by TLC. The reaction mixture was washed with 6 N aqueous HCl to protonate the thioacid product and to remove pyridine and propylamine. A single trailing spot beginning at R_f 0.1 was detected that corresponded to thiodecanoic acid. The thioacid was isolated by drying the hexane phase with anhydrous Na_2SO_4, followed by solvent evaporation.

Thioacids products were characterized by Fourier transform infrared (FTIR) spectroscopy and electron impact mass spectrometry (EI-MS); the respective spectra were acquired on Mattson Instruments Cygnus 25 FTIR (Madison, WI) and VG-TRIO1 (VG Instruments Co., Altrincham, England) spectrometers. The melting point of thiopalmitic acid was measured on a Laboratory Devices Melt-temp melting point apparatus and was not corrected.



SCHEME 1

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Abbreviations: BA, butylamine; EI-MS, electron impact mass spectrometry; FTIR, Fourier transform infrared; HPLC, high-performance liquid chromatography; PA, propylamine; RBF, round-bottom flask; TAA, thioacetic acid; TDA, thiodecanoic acid; THA, thiohexanoic acid; TLC, thin-layer chromatography; TMA, thiomyristic acid.

TABLE 1

Physical and Spectroscopic Data for Thioacid Products

Thioacid	Molecular formula	Formula weight	MS data[a]	Characteristic infrared frequencies
THA	$C_6H_{12}OS$	132.22		2563 cm^{-1} (SH) 1707 cm^{-1} (C=O)
TDA	$C_{10}H_{20}OS$	188.32	155 (−SH)	2561 cm^{-1} (SH) 1701 cm^{-1} (C=O)
TMA	$C_{14}H_{28}OS$	244.43	228 (−OH) 211 (−SH)	2564 cm^{-1} (SH) 1718 cm^{-1} (C=O)
TPA[b]	$C_{16}H_{32}OS$	272.49		2564 cm^{-1} (SH) 1716 cm^{-1} (C=O)

[a]Data are *m/e* values. Mass spectra (MS) were not acquired for thiohexanoic acid (THA) and thiopalmitic acid (TPA). TMA, thiomyristic acid; TDA, thiodecanoic acid.
[b]Melting point, 27–28°C. The other thioacids are colorless liquids.

RESULTS AND DISCUSSION

Table 1 lists spectroscopic data and other physical properties for the four fatty thioacids that were synthesized by the route shown in Scheme 1. Thiohexanoic (THA), TDA and thiomyristic acids (TMA) are liquids, and thiopalmitic acid is a low-melting solid. All the thioacids show C=O and S-H stretches in the FTIR spectra at 1700–1720 cm^{-1} and 2560–2565 cm^{-1}, respectively. These spectra lack the broad feature in the region 2500–3300 cm^{-1} expected for carboxylic acids, which demonstrates that the thioacids are not contaminated with the corresponding oxyacids. The EI-MS spectrum of TDA contains a peak at $m/e = 155$ for loss of SH, while the spectrum of TMA contains peaks at 211 and 228 for loss of SH and OH, respectively, from the parent ion.

The ease of the thioacid synthesis outlined herein is its greatest advantage. The use of highly toxic, foul-smelling reagents is avoided, and particular caution to exclude atmospheric moisture is not required. The synthesis is conducted at mild temperatures and takes only ten minutes. In addition, product workup is straightforward and quantitative yields are realized. Our procedure readily and inexpensively provides thioacid precursors for the enantiospecific synthesis of chiral sulfur-containing phospholipid, triacylglycerol and 1,2-diacylglycerol analogues (6).

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Lipids from the Paracloacal Glands of the Chinese Alligator (*Alligator sinensis*)

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Lipids from the paracloacal glands of adult Chinese alligators (*Alligator sinensis*) were analyzed by gas chromatography/mass spectrometry. Acetates, aliphatic alcohols, free fatty acids and waxes consisting primarily of hexadecanoates were indicated in the secretions of males and females. Cholesterol, a diterpene hydrocarbon, and a diterpene ketone were detected in secretions of males but not in those of females. Lipids identified in the paracloacal secretions of *A. sinensis* are compared with those reported from the American alligator and other crocodilians.

Lipids 28, 75–78 (1993).

All extant crocodilians possess paired exocrine organs, known as paracloacal glands, embedded in the walls of the cloaca (1). Behavioral observations of free-ranging adult crocodilians suggest that secretions discharged from these glands contain pheromones used in mating and/or nesting activities (2). Laboratory studies show that immature American alligators (*Alligator mississippiensis*) detect airborne paracloacal gland chemicals (2), but no specific pheromonal function for these exudates has been demonstrated.

Comparative analyses of paracloacal gland secretions by thin-layer chromatography (TLC) indicate a variety of lipid classes, as well as taxonomic and other possible sources of variation in secretion composition (3,4). Analyses by gas chromatography/mass spectrometry (GC/MS) revealed terpenes, cholesterol, free fatty acids, acetates and other lipids among the paracloacal gland products of the American alligator (5) as well as in the dwarf (*Paleosuchus palpebrosus*) and the smooth-fronted caimans (*P. trigonatus*) (6,7).

We report here the results of GC/MS analyses of lipids from the paracloacal glands of the Chinese alligator (*A. sinensis*), an endangered species from the lower Yangtze Valley in the Anhui and Zhejiang Provinces of the People's Republic of China (8). TLC of the paracloacal gland secretions of this species has previously indicated the presence of bands that can be attributed to sterols, free fatty acids and other lipid classes (3), but structural details of the compounds in these exudates have not been reported.

MATERIALS AND METHODS

Secretions from the paracloacal glands of *A. sinensis* were obtained in September, 1989 from four 9-year-old females at the Houston Zoological Gardens (Houston, TX) and in November 1989 from seven 4-year-old and three 9-year-old females and one 5-year-old and two 10-year-old males at the New York Zoological Park (Bronx, NY). All animals were hatched at the Rockefeller Wildlife Refuge in Grand Chenier, LA, and raised in captivity.

Each gland was manually compressed while secretions were directed into a vial held next to the everted gland duct opening. Approximately 3–5 mL of dichloromethane were added to each vial, and the vials were stored on ice for 48 h or less before being shipped on dry ice. Samples were kept at −10°C prior to analysis.

The paracloacal secretions of each individual sampled were placed separately into a glass tissue grinder and extracted three times with 25-mL vol of pesticide-grade hexane while they were macerated with a pestle. The hexane extracts were decanted from insoluble materials and combined in a separatory funnel. Extracts were separated into free fatty acid (FFA) and neutral lipid fractions by partitioning them with 75 mL of a saturated solution of $NaHCO_3$. The lower aqueous layer, which contained FFA, was acidified with 10M H_3PO_4 to pH 1 and extracted with 75 mL of diethyl ether/hexane (50:50, vol/vol). The extract was rinsed with distilled water and dried over anhydrous sodium sulfate.

Neutral lipids in the hexane layer were dried over anhydrous sodium sulfate and separated by TLC on 0.75-mm silica gel layers developed in hexane/ethyl acetate (95:5, vol/vol). TLC bands were visualized by spraying with a solution of Rhodamine B and dichlorofluorescein (9) and viewed under ultraviolet light. Bands were scraped from the plate, extracted with diethyl ether and analyzed by GC/MS.

FFA in the residue were converted to methyl esters by reacting them with CH_2N_2 in diethyl ether (10). They were then separated by TLC, as described above. Alcohols were converted to acetates by placing microgram quantities into a vial with 0.2 mL of acetic anhydride/pyridine (2:1, vol/vol). Waxes in the lipid mixture were saponified with alcoholic KOH and separated into alcohol and fatty acid fractions. Alcohols formed by saponification were acetylated; ethyl esters of the fatty acids were prepared with Ethyl-8 (Pierce Chemical, Rockford, IL).

Portions of the unsaturated lipids were hydrogenated in ether solution in a 5-mL Reactivial (Wheaton Scientific, Millville, NJ) using 50 mg of PtO_2 as a catalyst (T. Jones, personal communication). Hydrogen gas was bubbled through the solution at approximately 0.5 mL/min for 30 min. Dimethyl disulfide (DMDS) derivatives were prepared by placing approximately 1 μg of unsaturated lipid in approximately 50 μL of DMDS, along with a crystal of iodine (11,12).

GC/MS analyses were performed on a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph interfaced with a 5970 Mass Selective Detector (Hewlett-Packard). GC was done on a 30-m × 0.25-mm DB-1 fused silica column (J&W Scientific, Folsom, CA) using the following temperatures: injection port 250°C; transfer line, 280°C; oven, initially 50°C, then programmed to 150°C at 25°C/min, and then to 325°C at 4°C/min. The carrier gas was chromatog-

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Abbreviations: DMDS, dimethyl disulfide; FFA, free fatty acid(s); FTIR, Fourier transform infrared; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography.

raphy-grade helium maintained at a head pressure of 105 kPa. All spectra were obtained at an ionizing voltage of 70 eV.

Fourier transform infrared (FTIR) vapor-phase spectra were obtained on a Hewlett-Packard 5890 GC with a 5965A FTIR detector and a 7958 data system. A 30-m × 0.32-mm DB-5 column (J&W Scientific) using chromatographic conditions identical to those described above was used.

RESULTS

C_{14}–C_{18} FFA, with a predominance of C_{16} and C_{18} compounds, were characterized as methyl esters in the secretions of *A. sinensis* (Table 1). Pentadecanoic, *Z*-9-hexadecenoic and *Z*-9-octadecenoic acids were indicated in the secretions of females but not in those of males; males possessed a small amount (1%) of heptadecanoic acid, which was not detected in females.

TLC separation of neutral lipids from female *A. sinensis* revealed three major bands. The eluants of Band I (R_f 0.37) displayed GC retention times and mass spectra consistent with primary aliphatic alcohols, as confirmed by comparisons with authentic alcohols (NuChek Prep, Elysian, MN). The identification of alcohols was further verified by derivatization of TLC eluates with acetic anhydride/pyridine to form acetates that were analyzed by GC/MS (Table 1). The mass spectra of DMDS derivatives of monounsaturated acetates displayed characteristic methyl thioether ions, thus allowing double-bond positions to be assigned. Some ambiguous spectra, likely representing heterocyclic adducts formed from diunsaturated alcohols, were observed; interpretation of these spectra was confounded by the co-elution of two or more compounds.

Band II (R_f 0.51) consisted of a series of acetates with alcohol moieties similar to those of the free alcohols eluted from Band I (Table 2). The double-bond positions of the acetates were determined by adding DMDS and analyzing the resulting vicinal dithiomethyl ethers by GC/MS, as described above.

Band III (R_f 0.81) consisted of a complex mixture of waxes, the major components of which were esters of hexadecanoic acid (percent compositions in parentheses): heptyl (7%), octyl (12%), nonyl (4%), decyl (4%), undecyl (3%), dodecyl (2%), tridecyl (3%), tetradecyl (13%), pentadecyl (11%), hexadecyl (28%) and octadecyl (11%). The mass spectrum of each of these compounds contained the protonated hexadecanoate ion ($C_{15}H_{33}CO_2^+$, *m/z* 257) and a corresponding hydrocarbon fragment. MS ions characteristic of dodecanoates ($C_{11}H_{25}CO_2^+$, *m/z* 201), tridecanoates ($C_{12}H_{27}CO_2^+$, *m/z* 215), tetradecanoates ($C_{13}H_{29}CO_2^+$, *m/z* 229), pentadecanoates ($C_{14}H_{31}CO_2^+$, *m/z* 243), hexadecenoates ($C_{15}H_{28}CO^+$, *m/z* 236), heptadecanoates ($C_{16}H_{35}CO_2^+$, *m/z* 271), octadecenoates ($C_{17}H_{32}CO^+$, *m/z* 264) and octadecanoates ($C_{17}H_{37}CO_2^+$, *m/z* 285) also were observed. Compounds of the same molecular weight co-eluted, even on capillary columns, thereby yielding complex mass spectra and frustrating our attempts to identify and quantify the percent compositions of many of these components.

The percent compositions of alcohols, acetates and waxes in the paracloacal gland secretions of female *A. sinensis*, calculated from the weights of residues eluted from TLC plates, were 19, 72 and 9%, respectively.

TABLE 1

Free Fatty Acids and Alcohols from the Paracloacal Gland Secretions of *Alligator sinensis* Showing Typical Percent Compositions Within Each Compound Class for Males and Females

	% Composition	
	Male	Female
Fatty acids		
Tetradecanoic acid	5	trace
Pentadecanoic acid	0	6
Hexadecanoic acid	33	47
Z-9-Hexadecenoic acid	0	10
Heptadecanoic acid	1	0
Octadecanoic acid	61	16
Z-9-Octadecenoic acid	0	21
Alcohols		
3-Dodecen-1-ol	0	1
Dodecanol	0	1
4-Tridecen-1-ol	0	1
Tridecanol	2	2
5-Tetradecen-1-ol	4	5
Tetradecanol	6	4
x,y-Pentadecadien-1-ol	0	1
z,w-Pentadecadien-1-ol	0	2
x-Pentadecen-1-ol	0	2
8-Pentadecen-1-ol	0	3
x-Pentadecen-1-ol	0	1
Pentadecanol	14	8
x,y-Hexadecadien-1-ol	0	2
5-Hexadecan-1-ol	0	6
9-Hexadecen-1-ol	0	2
Hexadecanol	17	18
x,y-Heptadecadien-1-ol	0	1
z,w-Heptadecadien-1-ol	0	2
u,v-Heptadecadien-1-ol	0	4
8-Heptadecen-1-ol	22	5
10-Heptadecen-1-ol	0	1
Heptadecanol	5	0
5,9-Octadecadien-1-ol	0	8
x,y-Octadecadien-1-ol	0	5
9-Octadecen-1-ol	21	8
11-Octadecen-1-ol	0	3
Octadecanol	7	5

TLC separation of neutral lipids in the paracloacal gland secretions of male *A. sinensis* yielded six major bands. Band I (R_f 0.03) contained a single compound, cholest-5-en-3-ol (cholesterol). Band II (R_f 0.28) consisted of a phthalate ester, a plasticizer that belongs to a class of ubiquitous environmental contaminants, readily classified by a base peak at *m/z* 149. The presence of this compound in the paracloacal glands is curious, but it was not characterized further because we assumed that it is not of biological origin.

Band III (R_f 0.38) consisted of a mixture of saturated and mono- and diunsaturated primary alcohols similar to, but not as complex, as that observed from female alligators (Table 1). These compounds were identified by forming acetate derivatives that were analyzed by GC/MS, as described above. Likewise, DMDS derivatives were made of the unsaturated acetate derivatives to determine double-bond positions.

Band IV (R_f 0.66) was shown by MS and FTIR to contain a diterpene ketone with a molecular weight of 288 and a molecular formula of $C_{20}H_{32}O$. Band VI (R_f 0.91)

TABLE 2

Acetates from the Paracloacal Gland Secretions of Female *Alligator sinensis* Showing Typical Percent Compositions[a]

Compounds	% Composition
3-Dodecenyl acetate	1
Dodecyl acetate	1
4-Tridecenyl acetate	1
Tridecyl acetate	2
x,y-Tetradecadienyl acetate	1
5-Tetradecenyl acetate	4
Tetradecyl acetate	4
x,y-Pentadecadienyl acetate	1
z,w-Pentadecadienyl acetate	1
x-Pentadecenyl acetate	1
8-Pentadecenyl acetate	3
x-Pentadecenyl acetate	3
Pentadecyl acetate	8
x,y-Hexadecadienyl acetate	1
5-Hexadecenyl acetate	6
Hexadecyl acetate	20
4,8-Heptadecadienyl acetate	3
x,y-Heptadecadienyl acetate	3
z,w-Heptadecdienyl acetate	3
10-Heptadecenyl acetate	2
8-Heptadecenyl acetate	4
Heptadecyl acetate	1
5,9-Octadecadienyl acetate	7
x,y-Octadecadienyl acetate	7
9-Octadecenyl acetate	6
11-Octadecenyl acetate	1
Octadecyl acetate	3

[a]Trace amounts of pentadecenyl (10%), hexadecenyl (25%) and heptadecenyl acetates (65%) were detected in males.

contained a diterpene hydrocarbon that exhibited a molecular weight of 272 and a molecular formula of $C_{12}H_{32}$. Comparison of the FTIR spectra of the diterpene ketone and the diterpene hydrocarbon revealed identical absorbances, except for a carbonyl absorbance in the ketone at 1721 cm^{-1}.

Band V (R_f 0.81) consisted of a series of waxes that, as in females, was predominated by hexadecanoates (percent compositions in parentheses): dodecyl (2%), tridecyl (2%), tetradecyl (16%), pentadecyl (47%), hexadecyl (25%), heptadecyl (1%) and octadecyl (7%). MS ions characteristic of dodecanoates, tridecanoates, tetradecanoates, pentadecanoates, heptadecanoates, octadecenoates and octadecanoates also were observed; in contrast to the results with females, however, hexadecenoates were not detected in males.

The percent compositions of cholesterol, alcohols, the diterpene ketone, waxes and the diterpene hydrocarbon in the paracloacal gland secretions of male *A. sinensis*, calculated from the weights of residues eluted from TLC plates, were 1, 10, 15, 53 and 2%, respectively.

DISCUSSION

A TLC survey of 21 crocodilian species suggested the widespread occurrence of FFA in the paracloacal gland secretions (3). C_{16} and C_{18} FFA from the secretions of the American alligator and the dwarf caiman have been elucidated previously by GC/MS (5,7). Saturated and unsaturated C_{14}–C_{21} fatty acids also have been reported in the skin gland secretions of crocodiles (*Crocodylus* spp.), however, the exudates analyzed included secretions pooled from both the paracloacal glands and the gular glands (paired exocrine organs on the lower jaw) (13,14); thus, unfortunately, the source of the FFA is unclear. Our analysis indicates C_{14}–C_{18} FFA among the paracloacal products of *A. sinensis*. Cholesterol, another common tetrapod skin product, also was detected among the paracloacal gland secretions of male alligators.

A number of aliphatic alcohols have been documented among the paracloacal products of caimans (*Caiman* spp. and *Paleosuchus* spp.) (7,15,16), but these compounds were not reported for an analysis of the secetions of the American alligator (5). Our investigation indicates C_{12}–C_{18} saturated and unsaturated aliphatic alcohols in *A. sinensis*, thus establishing the presence of this compound class in the paracloacal secretions of the genus *Alligator*. 3-Dodecen-1-ol, dodecanol and an array of unsaturated C_{15}–C_{18} primary alcohols were detected in females, but not in males.

Acetates ranging in carbon-chain length from C_{10} to C_{18} were indicated in the paracloacal gland secretions of American alligators of both sexes and, to a lesser extent, in immature (unsexed) individuals (5). Our analysis of *A. sinensis* indicates C_{12}–C_{18} acetates primarily in females. C_{15}, C_{16} and C_{17} monounsaturated acetates in the total lipid mixture of some males were indicated by MS ions at *m/z* 61 and molecular ions 60 mass units less than the molecular weight (M−60); the unsaturation sites of these compounds were not determined because only trace amounts of these compounds were present and they were not detected by TLC. A number of mono- and diunsaturated compounds not detected in the American alligator were indicated in *A. sinensis*.

Dodecanoates, tetradecanoates and other waxes were observed in the paracloacal gland secretions of immature American alligators (5). Our results indicate a complex mixture of waxes in the secretions of adult *A. sinensis*, but detailed identifications were made only for hexadecanoates. The hexadecanoates indicated in female *A. sinensis* secretions range in carbon-chain length from C_7 to C_{18} whereas those of males range from C_{12} to C_{18}. Waxes comprised a larger percentage of the total lipids of the secretions of males than those of the females (53% *vs.* 9%, respectively).

A recent study of the smooth-fronted caiman indicates (*E,E*)-7,11,15-trimethyl-3-methylenehexadeca-1,6,10,14-tetraene (β-springene) in the paracloacal gland secretions (6). Traces of other diterpenes and β-farnesene also were detected. The diterpene hydrocarbon observed in male *A. sinensis* has the same molecular weight as β-springene, but the mass spectra of these two compounds are different. Carbon-13 and proton nuclear magnetic resonance data are needed to determine the structure of the compound in *A. sinensis* and that of an apparently related ketone.

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ERRATUM

In *Lipids*, Vol. 27, no. 11 (November 1992), "Bile Salt Stimulated Lipase: Comparative Studies in Ferret Milk and Lactating Mammary Gland," Ellis, L.A., and Hamosh, M., pp. 917-922, columns A and B of Figure 2 on page 919 were inadvertently omitted. The entire figure is reproduced below.

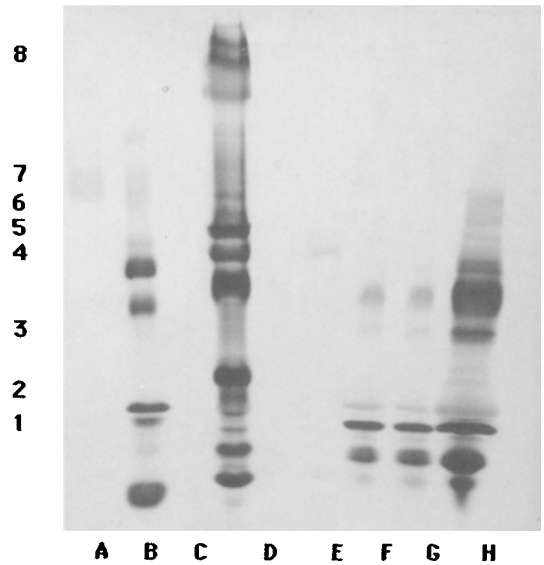

FIG. 2. Sodium dodecyl sulfate polyacylamide gel electrophoresis of purified ferret and human milk bile salt stimulated lipase (BSSL). Lanes: A, BSSL purified from ferret milk; B, BSSL purified from human milk; C, human milk, skim milk fraction; D, standards for molecular weight; E, BSSL purifed from ferret milk; F, ferret milk casein pellet, second centrifugation; G, ferret milk casein pellet, first centrifugation; H, ferret milk, skim milk fraction. Standards for molecular weight (kD), lane D: 1, lysozyme (14.4 kD); 2, trypsin inhibitor (22 kD); 3, carbonic anhydrase (31 kD); 4, ovalbumin (45 kD); 5, serum albumin (66 kD); 6, phosphorylase B (97 kD); 7, β-galactosidase (116 kD); 8, myosin (200 kD).

ARTICLE

Overexpression of a *Rhizopus delemar* Lipase Gene in *Escherichia coli*[1]

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A cloned complementary deoxyribonucleic acid encoding the precursor polypeptide of an extracellular lipase from the fungus *Rhizopus delemar* was altered by site-directed mutagenesis to generate deoxyribonucleic acid fragments that specifically code for the polypeptides of the proenzyme and the mature form of the lipase. Attempts to produce these polypeptides in enzymatically active form in *Escherichia coli* revealed toxic effects toward the host. Therefore the polypeptides were expressed as inactive and insoluble forms in the cytoplasm of *E. coli* BL21 (DE3) cells using plasmid vector pET11-d. With this tightly regulated high-level expression system, lipase and prolipase polypeptides were produced to estimated levels of up to 21% and 15%, respectively, of total cellular protein. The insoluble polypeptides were solubilized in 8 M urea. Refolding into active forms was achieved by treatment with the redox system cystine/cysteine and dilution. Refolded mature lipase was purified to homogeneity by affinity and ion exchange chromatography. The enzyme had a specific activity comparable to that of lipase from the fungal culture. The quantities of pure enzyme obtained from a 1-L culture of *E. coli* exceeded those obtained from the fungal culture by a factor of at least 100. Refolded recombinant prolipase was purified essentially to homogeneity and had a specific activity similar to that of the mature enzyme. Its pH optimum was 7.5, rather than the pH 8 determined for recombinant mature lipase and for the enzyme purified from the fungal culture. Recombinant prolipase retained activity after 15 min incubation at 65°C, while mature lipase retained activity only up to 45°C.
***Lipids* 28, 81-88 (1993).**

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) are enzymes capable of hydrolyzing ester bonds of water-insoluble substrates at the substrate-water interface. Lipolytic enzymes are ubiquitous and their physical and biochemical diversity provides the basis for many current and future biotechnological uses. In nature, the hydrolysis of acylglycerols is probably the main function of lipases. Under laboratory conditions, lipases are able to catalyze transesterification and ester synthesis reactions with alcohols other than glycerol and to hydrolyze a diversity of esters in nonaqueous solvent systems (1).

The fungus *Rhizopus delemar* (presently designated *R. oryzae*) produces extracellular lipases that hydrolyze the ester bonds at the *sn*-1 and *sn*-3, but not the *sn*-2 position, of a triacylglycerol (2). This pronounced positional specificity is one of the reasons for continued basic studies on the *R. delemar* lipases and for the use of partially purified *R. delemar* enzyme preparations in acylglycerol restructuring (3), the exchange of acyl groups of phospholipids (4) and ester and glyceride synthesis (5-7).

Industrial applications of lipases and basic scientific experiments are often hampered by the lack of sufficient enzyme or by the heterogeneity of the available preparations. The complete purification of the *R. delemar* lipase is time-consuming and yields only about 0.2-0.6 mg per liter of culture supernatant (8). Low purification yields have prompted researchers to employ molecular cloning techniques to improve the production of lipases. For example, the complementary DNA (cDNAs) for extracellular lipases from *Rhizomucor miehei* and *Humicola lanuginosa* were overexpressed in the fungus *Aspergillus oryzae*, and mature lipase was purified from the culture medium (9,10). The gene for a thermostable lipase from the bacterium *Pseudomonas fluorescens* was overexpressed in *Escherichia coli* to a level of 40% of the total protein (11).

The use of recombinant DNA techniques for the production of an *R. delemar* lipase became possible after a cDNA clone of the lipase was obtained and its nucleotide sequence was determined (12). The sequence data predict that the fungus produces a mRNA that is translated into a preproenzyme. The export signal peptide and the propeptide are then removed proteolytically to produce the mature lipase enzyme. Here we report on the cloning of DNAs encoding the prolipase and the mature lipase in *E. coli*, their expression in the bacterium in inactive form, their refolding *in vitro* to active lipolytic proteins, and their purification.

MATERIALS AND METHODS

DNA manipulations. Plasmid DNA and phage M13 RF DNA were isolated by an alkaline lysis procedure (13) followed by a phenol/chloroform extraction. Restriction enzyme digestions and ligation reactions were performed under conditions recommended by the suppliers. Transformation with plasmid DNA was accomplished using $CaCl_2$-treated *E. coli* cells as described by Sambrook *et al.* (14).

Construction of lipase expression plasmids. The previously cloned cDNA encoding the *R. delemar* lipase (12) was excised from plasmid pUC8-2.14 (12) as an *Eco*RI fragment and inserted into the *Eco*RI site of bacteriophage M13 mp18 (15). Site-directed mutagenesis of the insert DNA was carried out according to the method of Kunkel (16) using the MUTA-GENE M13 *IN VITRO* MUTAGENESIS KIT (Bio-Rad Laboratories, Richmond, CA). Mutagenic primers were obtained from the Department of Chemistry, University of Pennsylvania, Philadelphia, PA. A primer, 5'GGTTCATCCATGGTTCCTGT 3', was used to replace the codon for the C-terminal amino acid, alanine, of the predicted signal peptide of preprolipase with a codon representing methionine. This primer also changes the nucleotide sequence to introduce an *Nco*I

[1]Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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Abbreviations: bp, base pairs; cDNA, compementary deoxyribonucleic acid; CM-Sepharose, carboxymethyl Sepharose; DNA, deoxyribonucleic acid; DTT, diethiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; OA, oleic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; U, units of lipase activity.

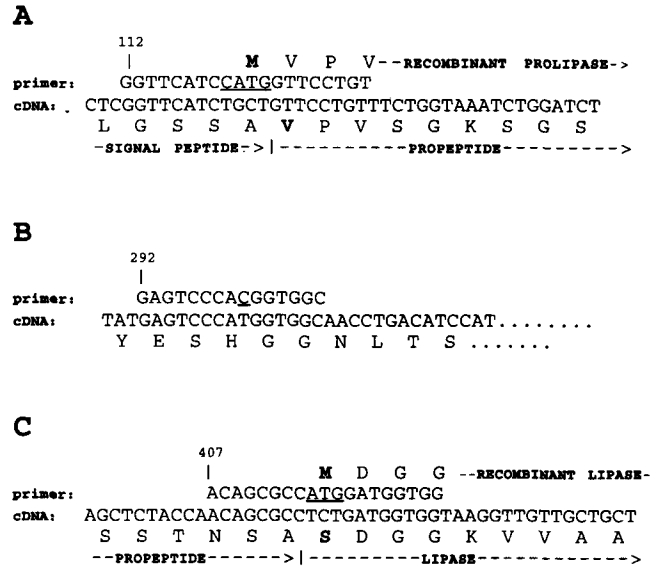

FIG. 1. Oligonucleotide primers used for mutagensis of the *R. delemar* lipase cDNA. A. Primer for the introduction of a methionine codon and an *Nco*I site at the position in the *R. delemar* lipase cDNA encoding the junction between signal peptide and propeptide. B. Primer for the removal of an *Nco*I site within the propeptide encoding region. C. Primer for the introduction of a methionine codon and an *Nco*I site at the position in the lipase cDNA encoding the junction between propetide and mature lipase. The amino acid sequence predicted from the cDNA is given below the nucleotide sequence. The amino acid sequence resulting from the oligonucleotide directed mutagenesis is given above the primer sequence. The numbers indicate positions of nucleotides in the published nucleotide sequence of the *R. delemar* cDNA (ref. 12).

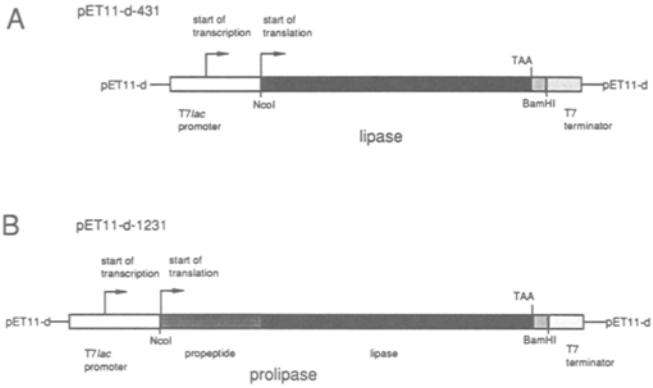

FIG. 2. Schematic representation of the lipase- and prolipase-encoding segments in the expression plasmid pET11-d (ref. 18). The lipase and prolipase genes were cloned as *Nco*I-*Bam*HI fragments between the T7*lac* promoter and T7 terminator sites in plasmid pET11-d. The termination codon for lipase and prolipase is indicated by the nucleotide triplet TAA.

site (Fig. 1A). An already existing *Nco*I site within the coding region was removed using a primer 5′GAGTCCCACGGTGGC 3′ (Fig. 1B). This base change did not alter the predicted amino acid sequence, but replaced the histidine codon, CAU, with the histidine codon, CAC, found considerably more frequently in mRNA from highly expressed genes of *E. coli* (17). Both primers were employed in a single mutagenesis experiment. In a second mutagenesis, a primer, 5′ACAGCGCCATGGATGGTGG 3′, was used to replace the codon for the *N*-terminal amino acid serine of the mature lipase enzyme (12) with the codon representing methionine (see Fig. 1C). This change also introduced an *Nco*I site. Phage carrying mutagenized cDNA inserts were identified by restriction enzyme cleavage of their double-stranded DNA. *Nco*I-*Bam*HI fragments, approximately 800 base pairs (bp) and 1100 bp in size, representing lipase- and prolipase-encoding DNA respectively, were isolated and ligated to vector pET11-d (18) that had been cleaved with *Nco*I and *Bam*HI (Fig. 2). The resulting plasmids, pET11-d-431 (Fig. 2A) and pET11-d-1231 (Fig. 2B), were introduced into *E. coli* BL21 (DE3) (18) by transformation.

Induction of lipase gene expression. E. coli BL21 (DE3) harboring the pET11-d recombinant plasmids were grown on solid LB agar medium (19) containing ampicillin (100 μg/mL). Cells from a single colony were inoculated into LB broth (19) containing ampicillin (100 μg/mL) and grown overnight at 30°C with light shaking. The culture was then diluted 100-fold into medium made up of the components of LB and of M9 minimal medium (19), pH 7.4, containing ampicillin (200 μg/mL). The cultures (500 mL of medium in 2 L baffled flasks) were shaken vigorously at 37°C until the optical density at 600 nm reached a value between 0.6 and 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. Incubation was resumed, and the cells were harvested by centrifugation 3 to 4 h after the addition of IPTG. For experiments using lactose as inducer, the sugar was added to a final concentration of 10 mM.

Refolding of lipase. The cells obtained from a 1-L culture were resuspended in 50 mL of 50 mM Tris-HCl, pH 8, 5 mM ethylenediaminetetraacetic acid (EDTA), 10% sucrose. Lysozyme was added to a final concentration of 0.8 mg per mL. The mixture was incubated for 30 min at room temperature. Four-hundred mL of Triton buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% Triton X-100) were added. After mixing, the lysate was sonicated to reduce viscosity and centrifuged at 15,000 × *g* for 30 min at 4°C. The insoluble pellet was resuspended in 400 mL of the Triton buffer and sonicated briefly. The resulting suspension was centrifuged at 15,000 × *g* for 20 min. The pellet was resuspended in 400 mL of 10 mM Tris-HCl, pH 8, 1 mM EDTA, and the suspension was centrifuged as above. The pellet was finally washed with distilled water and resuspended in 10 mL of distilled water. This suspension was added to 200 to 400 mL of 8 M urea [previously deionized by passage through mixed-bed resin AG 501-X8 (Bio-Rad Laboratories, Hercules, CA)] in 25 mM sodium phosphate, pH 7, 1 mM EDTA, 5 mM dithiothreitol (DTT). The solution was incubated at room temperature for 1 h, then centrifuged at 15,000 × *g* for 30 min at room temperature. Cystine (dissolved in 0.6 N NaOH) was added to a final concentration of 15 mM. The pH of the solution was maintained at 8.5 to 9 for 10 min. Then the solution was slowly added to 10 to 20 vol of cold (4–10°C) 50 mM sodium phoshate, pH 7.5–8, 1 mM EDTA, 5 mM cysteine. This solution was incubated for at least 48 h at 4°C to allow refolding of the denatured protein.

Purification of lipase and prolipase. The pH of the refolding solution was adjusted to 6.5 for lipase and 6.0 for prolipase by the dropwise addition of H_3PO_4. The solu-

tions were filtered through Whatman No. 1 paper (Whatman International Ltd, Maidstone, England) and concentrated in a stirred ultrafiltration cell using Diaflo ultrafiltration membranes YM10 or YM30 (Amicon, Beverly, MA) or by filtration through Minitan ultrafiltration filterplates type PLGC (Millipore, Bedford, MA).

The concentrated solution containing refolded lipase was filtered through Whatman No. 1 filter paper and loaded onto an oleic acid (OA) affinity chromatography column (16 × 250 mm) prepared as described previously (8). The column was washed with 200 mL 20 mM sodium phosphate, pH 6.5, 5% NaCl, followed by 200 mL 20 mM sodium phosphate, pH 6.5. The lipase was eluted with a gradient of Triton X-100 (0–0.5%) in 20 mM sodium phosphate, pH 6.5. For applications where the presence of detergent was not desirable, 60% ethyleneglycol in 20 mM sodium phosphate buffer, pH 6.5 was used for elution. Fractions containing lipase activity in excess of 200 U/mL were pooled and loaded onto a 12 × 200 mm column of carboxymethyl-sepharose (CM-Sepharose) CCL-6B-100 (Sigma, St. Louis, MO) equilibrated with 20 mM sodium phosphate, pH 6.5. The column was washed with 40 mL of the same buffer, and the lipase was eluted with 200 mL of an NaCl gradient (0–500 mM) in 20 mM sodium phosphate, pH 6.5.

Refolded prolipase did not bind to the oleic acid affinity gel. The buffer of the concentrated refolding solution was therefore exchanged for 20 mM sodium phosphate, pH 6, by diafiltration, and the solution was loaded onto a CM-Sepharose column (12 × 200 mm) equilibrated with 20 mM sodium phosphate, pH 6. The prolipase was eluted with a 0–500 mM NaCl gradient in the same buffer.

General protein techniques. Sodium dodecylsulfate (SDS)-12.5% polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (20). The molecular weight of proteins was estimated by comparison of their mobilities with those of protein standards (Dalton Mark VII-L, Sigma). Protein bands on gels were stained with Coomassie Brilliant Blue R. An estimate of the relative intensity of bands on dried Commassie Blue-stained gels was obtained by scanning with a Bio-Rad Model 620 Video Densitometer (Bio-Rad, Richmond, CA) and analysis of the scanning profile with the Bio-Rad 1-D Analyst II programs.

Protein concentrations were determined by the method of Bradford (21) using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Bovine-γ-globulin was used as the standard.

N-Terminal amino acid sequences of recombinant lipase and prolipase were determined by automated Edman degradation using an Applied Biosystem (Foster City, CA) 473A Protein Sequencer. Determinations were made from 300 pmoles of protein dissolved in 0.1% trifluoroacetic acid.

Determination of lipase activity. Activity was determined qualitatively by spotting samples onto solid media containing olive oil (25 mL/L) emulsified in 20 mM sodium phosphate, pH 7.5, 0.001% Rhodamine B (Sigma). Lipase activity was visualized under ultraviolet light (22). Quantitative activity determinations were carried out titrametrically as described previously (8) using a VIT 90 Video titrator (Radiometer, Copenhagen, Denmark). The pH of the reaction mixture was maintained by the continuous addition of 0.1 N NaOH. For routine activity measurements, the assays were conducted at 25 °C at pH of 7.5 using purified olive oil (Sigma) as substrate. A thermostatted, water-jacketed reaction chamber was used for the determination of the effect of temperature on the lipase activity. Lipase activity was calculated from the maximum rate of titrant addition using a lipase titrametric assay program (Cichowicz, D.J. *et al.*, unpublished). A unit (U) of activity released one μmole of fatty acid per minute.

RESULTS AND DISCUSSION

Expression of recombinant lipase and prolipase. Over the years, *E. coli* has proven to be a suitable and often preferable organism for the overexpression of both pro- and eukaryotic genes. The ability of this bacterium to synthesize lipolytic protein from *R. delemar* cDNA cloned in lambda gt11 was crucial in the original isolation of the lipase cDNA (12). This cDNA was subsequently cloned in plasmid pUC8-2 (23), and, after addition of IPTG, *E. coli* cells harboring the resulting recombinant plasmid pUC8.2-14 (12) produced lipolytic protein with the same positional specificity as that of lipase produced by the fungus (12). However, only small amounts of lipolytic protein were present in cell-free extracts from induced recombinant *E. coli*, and the protein species that cross-reacted with polyclonal antibody raised against the fungal lipase were all larger than the 30 kDa molecular mass of mature *R. delemar* lipase (8,12). This indicated that *E. coli* JM101 was unable to fully process precursor proteins. Therefore, to obtain recombinant lipase corresponding to the mature fungal enzyme, it was necessary to remove the portions of the *R. delemar* lipase cDNA that encode the pre- and propeptide fragments (Fig. 1C). A DNA fragment encoding prolipase was also generated by removal of the part of the cDNA encoding the fungal signal peptide (Fig. 1A). The availability of a prolipase clone was desirable, because it was unknown if the propeptide was required for or beneficial to obtaining active lipase.

Many factors are known to determine the ultimate level of expression of a cloned gene. Among those factors are transcription rate and stability of the mRNA, rate of translation and stability of the protein product and also the amount of protein that can be accumulated before toxic effects occur. Attempts to clone the *R. delemar* lipase cDNA, excised from pUC8-2.14, into expression vectors pRX-1 (24) allowing transcription from a *trp* promoter or into the pMAL vectors (New England Biolabs, Beverly, MA) allowing the production of MaLE fusion proteins after transcription from a *lac* promoter, demonstrated that the presence of the cDNA in the correct orientation with respect to the promoters lead to plasmid instability and impaired viability even in the absence of inducer substances (data not shown). Thus the protein species produced from the cDNA have toxic properties which could lead to the selection of recombinant strains with low expression levels. The toxicity might be caused by the presence of the fungal signal peptide in the recombinant polypeptides or by lipolytic activity of the protein species produced from the cDNA. As mentioned above, the mutagenesis outlined in Figure 1 allowed the isolation of lipase- and prolipase-encoding DNAs that lack the sequences coding for the fungal signal peptide. To further improve the chances for successful expression of these DNAs in

E. coli, an expression system was sought that would provide a tighter control over transcription than either *trp* or *lac* promoters could provide. Such a system should prevent the appearance of damaging levels of lipolytic recombinant protein during the growth phase of the cells in the absence of an inducing substance.

Studier *et al.* (18) developed an *E. coli* expression system that included an additional layer of gene control. The vector components (pET plasmid series) place the transcription of insert DNA under the control of a bacteriophage T7 gene 10 promoter. This promoter is recognized by T7 RNA polymerase, which is in turn regulated by a *lac* promoter in the host component of this expression system, *E. coli* BL21 (DES3) (18). The activity of low levels of T7 RNA polymerase synthesized under noninducing conditions is inhibited by the presence of intracellular lysozyme produced in strain *E. coli* BL21 (DE3)[pLysS] (18). Using a derivative of this system, Deng *et al.* (25) were able to overexpress bovine pancreatic phospholipase A_2, an enzyme toxic to *E. coli*. The phospholipase accumulated in the periplasm of *E. coli* since the phospholipase gene had been fused to DNA encoding the OmpA signal peptide from *E. coli*. We attempted to express the *R. delemar* lipase and prolipase genes using the system employed by Deng *et al.* (25). However, recombinant lipase and, to a lesser extent, recombinant prolipase were still toxic to *E. coli* BL21 (DE3)[pLysS].

The toxic effect of the expressed lipase, which manifested itself even under noninducing conditions, necessitated a more tightly controlled expression system for recombinant *R. delemar* lipase. Therefore plasmid pET11-d (18) was selected. It is similar to the vector of the expression system described above, but it contains, as additional control features, a *lac* operator site (required for *lac* repressor binding) located in the T7 promoter region and also the gene encoding the *lac* repressor. The lipase and prolipase genes, on their respective *Nco*I-*Eco*RI fragments, were cloned into one of these plasmids, vector pET11-d (18) (Fig. 2). *E. coli* BL21 (DE3) cells containing the recombinant plasmids were stable under noninducing conditions. Upon induction with IPTG, the cells accumulated protein that migrated to positions on SDS-PAGE corresponding to the predicted molecular weights of prolipase or lipase (Figs. 3 and 4). Scanning of gels indicated that recombinant lipase constituted between 15 and 21% of the total cellular protein. Extracts from cells producing recombinant prolipase contained between 9 and 15% prolipase.

Lactose can be used as a substitute for the relatively expensive inducer substance IPTG. As was observed earlier with *E. coli* HB101 harboring a recombinant plasmid containing a *tac* promoter (26) and with *E. coli* BL21 harboring a recombinant pET3 plasmid (27), the appearance of recombinant protein was delayed by approximately one hour when lactose was added instead of IPTG. However, Neubauer *et al.*(27) demonstrated that lactose can be as efficient an inducer as IPTG provided that the addition of lactose was timed with respect to the glucose level in the growth medium. Therefore, lactose might be a suitable inducer for lipase-producing *E. coli* strains grown in fermentors equipped with glucose biosensors.

Lysates of cells containing the pET11-d derivatives and expressing the recombinant lipase or prolipase exhibited very little, if any, lipase activity (Tables 1 and 2). After low-

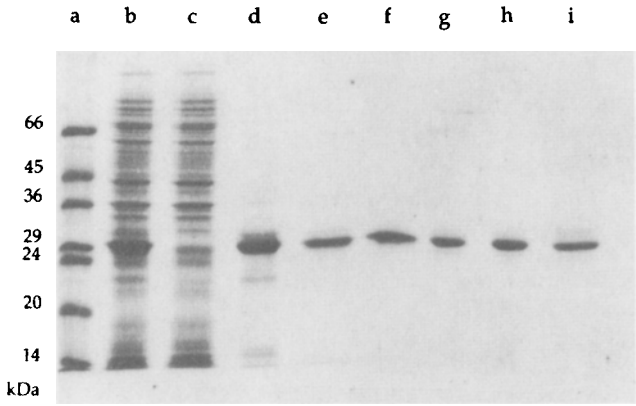

FIG. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of protein samples obtained during refolding and purification of recombinant *R. delemar* lipase. Lane a, molecular weight markers; lane b, lysate of *E. coli* BL21 (DE3) harboring pET11-d-431, induced for lipase expression; lane c, soluble fraction of lysate; lane d, buffer-insoluble fraction of lysate, dissolved in 8 M urea; lane e, concentrated refolding mixture to be subjected to affinity chromatography; lane f, combined fractions from affinity chromatography that exhibited significant lipase activity; lanes g and h, lipolytically active fractions eluted from CM-Sepharose column; lane i, *R. delemar* lipase purified from the fungal culture medium.

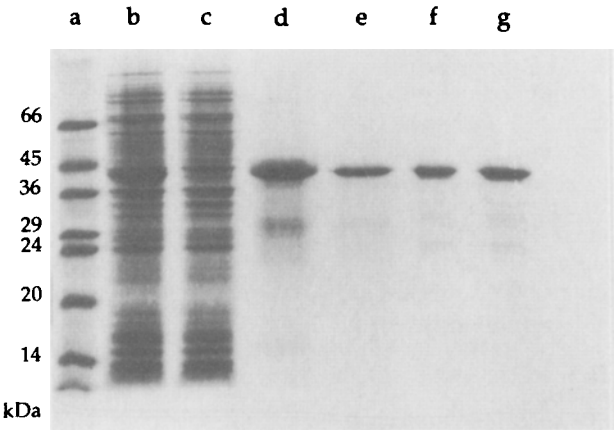

FIG. 4. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of protein samples obtained during refolding and partial purification of recombinant *R. delemar* prolipase. Lane a, molecular weight markers; lane b, lysate of *E. coli* BL21 (DE3) harboring pET11-d-1231, induced for prolipase expression; lane c, soluble fraction of lysate; lane d, buffer-insoluble fraction of lysate, dissolved in 8 M urea; lane e, concentrated refolding mixture, to be subjected to ion exchange chromatography on CM-Sepharose; lanes f and g, lipolytically active fractions eluted from CM-Sepharose.

speed centrifugation of cell lysates, the supernatant appeared to be devoid of the IPTG-inducible protein (Fig. 3, lane c), which was now found in the pellet. This is indicative of protein that has aggregated as inclusion bodies, a phenomenon often encountered with high expression systems (28). The highly expressed polypeptides remain in unfolded or partially folded states, and their sulfhydryl groups are mostly reduced (29). In this inactive state, lipase and prolipase polypeptides did not appear to cause cell lysis during the three- to four-hour induction period.

Refolding of recombinant lipase. The appearance of pro-

TABLE 1

Refolding and Purification of Recombinant *R. delemar* Lipase

Step	Protein[a] (mg)	Activity[b] (U)	Specific activity (U/mg)
Soluble fraction of lysate	1243	n.d.[c]	—
Insoluble fraction of lysate dissolved in 8 M urea	308	n.d.	—
Refolding solution (concentrated and filtered)	152	402234	2646
Combined fractions from oleic acid affinity column	104	397420	3821
Peak fractions CM-Sepharose column	62	315840	5094

[a]Bio-Rad protein assay.
[b]Assayed titrametrically. Units: μmole fatty acid released per min.
[c]None detected.

TABLE 2

Refolding and Purification of Recombinant *R. delemar* Prolipase

Step	Protein[a] (mg)	Activity[b] (U)	Specific activity (U/mg)
Soluble fraction of lysate	1246	n.d.[c]	—
Insoluble fraction of lysate dissolved in 8 M urea	170	n.d.	—
Refolding solution (concentrated and filtered)	117	429408	3670
Peak fractions CM-Sepharose column	59	373678	6333

[a]Bio-Rad protein assay.
[b]Assayed titrametrically. Units: μmole fatty acid released per min.
[c]None detected.

tein in insoluble form makes it necessary to devise a way to solubilize the polypeptides and to induce them to refold into an active form. The *R. delemar* lipase contains six cysteine residues which, based on amino acid sequence similarity with the lipase from *Rhizomucor miehei* (30,31), probably form three disulfide bridges. Therefore, refolding of recombinant *R. delemar* lipase has to include the formation of the proper disulfide bonds. The procedures necessary for successful refolding potentially add to the effort required for obtaining a pure protein, but there are advantages as well when protein is produced as inclusion bodies. First, a protein fraction highly enriched in the recombinant protein is obtained simply by separating the soluble from the insoluble fraction of a lysate. Second, recombinant protein is often synthesized in considerably higher amounts when deposited in inclusion bodies than when produced as soluble product. Thus, as shown for recombinant bovine interferon (32), the yield of active recombinant protein from cells producing inclusion bodies might be greater than that obtained from cells producing the recombinant protein in soluble form, even if only about 10% of the insoluble protein could be refolded properly.

The lipase-containing inclusion bodies were solubilized with 8 M urea, and unwanted inter- or intramolecular disulfide bonds that might have formed within the host cells or during isolation of the inclusion bodies were reduced with 5 mM DTT. In the resulting solution, 61 to 85% of the protein present co-migrated with purified *R. delemar* lipase on SDS-PAGE. Refolding was conducted on this solution of crude urea-solubilized inclusion bodies. The refolding procedure that gave the highest yield of refolded lipase was similar to that described for secretory leukocyte protease inhibitor (33). First, the urea-solubilized proteins were oxidized by the addition of cystine at a pH of 8.5 to 9. Then the urea concentration was reduced 10- to 20-fold by dilution to allow the folding process to commence. The dilution buffer contained cysteine to catalyze disulfide bond interchange. A pH of 7.5 to 8 in the dilution buffer gave the highest yield of active lipase.

During refolding, lipase displayed a tendency to aggregate and precipitate. Protein concentrations had to be kept below 100 μg/mL when diluting the urea-denatured protein in order to minimize this phenomenon. As was observed with other proteins (34,35), low temperatures during dilution also reduced the loss due to aggregation. The highest yield of active enzyme was achieved in refolding experiments conducted at 4 to 10°C. The yield was lower when the dilution buffer was at room temperature, and no active enzyme was obtained in experiments conducted at 37°C.

Refolding in buffer of pH 7.5 to 8 at 4°C was a relatively slow process and required several days for completion (Fig. 5). Some precipitate appeared during this time in the refolding solution. It was removed by filtration through Whatman No. 1 filter paper. In order to avoid strong precipitation during subsequent ultrafiltration and purification steps, the pH of the refolding solution was lowered to 6.5.

Purification of recombinant lipase. The mature recombinant lipase was purified by oleic acid affinity and ion

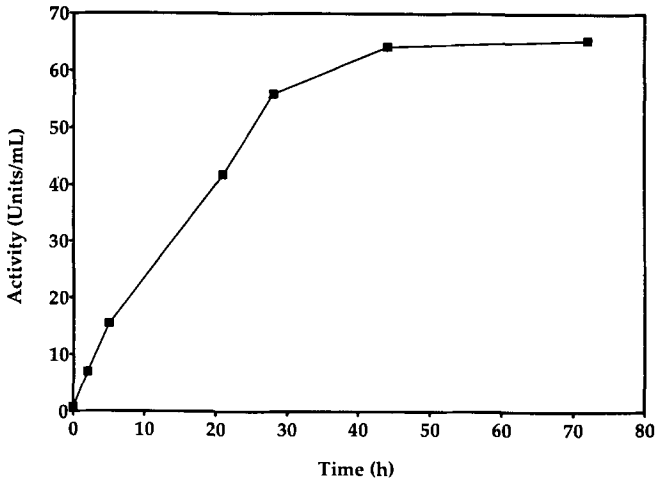

FIG. 5. Appearance of lipase activity after reduction of urea concentration from 8 M to 0.4 M. Samples were withdrawn from the refolding solution at the indicated time points and assayed for lipase activity as described in Materials and Methods.

exchange chromatography steps similar to those used for purification of the fungal enzyme (8). The activity recovered from 1 L of culture was almost completely retained by the 16 × 250 mm oleic acid affinity column. This activity was eluted virtually quantitatively as a single peak at a Triton X-100 concentration of approximately 0.25%. For purposes such as crystallization of the purified lipase, the presence of Triton X-100 might be undesirable due to its ability to bind to hydrophobic portions on proteins. Therefore an agent that presumably would not bind tightly to protein was sought to elute lipase from the OA affinity column. Ethylene glycol (60%) successfully eluted lipase from the column. The activity emerged in a broad peak, and trailing of activity was observed. Partially pure enzyme, recovered from the OA column either with Triton X-100 or ethylene glycol, was purified to apparent homogeneity by chromatography on CM-Sepharose at pH 6.5. (Fig. 3, lanes g–i). Migration of purified recombinant lipase on SDS-PAGE gels under reducing and nonreducing conditions was identical to that of lipase purified from *R. delemar*. The specific activity of fractions obtained after the ion exchange chromatography step was between 5000 and 8000 U per mg of protein. Similar specific activities were reported for purified lipase from *R. delemar* (8).

Amino acid sequence determination revealed that the *N*-terminal methionine was not removed post-translationally from the recombinant lipase. The second amino acid was predicted to be an aspartate (12); however, two sequencing runs failed to unambiguously discern between aspartate or asparagine as the identity of this residue. The sequence of the following 18 amino acids was identical to that determined for purified fungal-produced lipase and that predicted from the nucleotide sequence of the cDNA (12).

Refolding and partial purification of recombinant prolipase. Based on nucleotide sequence data (12), the *R. delemar* lipase was predicted to be synthesized as a proenzyme. The reason for the existence of the propeptide in lipase precursors is not known. Possibly, propeptides could be inhibitors of lipase, preventing membrane autolysis while the lipase is located inside the cell. This role for the propeptide was suggested for prophospholipase because the proenzyme did not exhibit interfacial activation and acted only on monomeric substrate (36). Thus prophospholipase might be unable to damage cellular phospholipid-containing structures.

It is also possible that the proregion is required for proper folding of the mature enzyme, as was observed for the *in vitro* and *in vivo* folding of some proteases (37,38). However, our data demonstrate that the propeptide is not required for *in vitro* refolding of mature recombinant *R. delemar* lipase. Whether or not the propeptide is involved in folding events *in vivo* or in some conformational changes during secretion from the fungal cell is unknown.

In vitro refolding of urea-denatured partially purified prolipase was conducted using the conditions used for the refolding of mature lipase. Yields from a refolding and purification experiment are shown in Table 2. The appearance of lipolytic activity measured titrametrically followed the same time course as that for lipase, and similar amounts of total activity were obtained from crude preparations of both proteins. Therefore the propeptide did not act as an inhibitor of recombinant lipase under the conditions employed. However, refolded recombinant prolipase behaved differently from recombinant mature lipase on solid media containing rhodamine B. Development of fluorescence was slower than was observed for the mature lipase, and fluorescent material was found in a diffuse zone around the site of sample application rather than only at the site of sample application. It is not known whether this was caused by physical differences, *e.g.*, mobility of the proteins in the agar medium, or by reactive differences that cause the differential removal of substrate or the generation of different products.

When purification was attempted in the same manner as for refolded recombinant lipase, recombinant prolipase did not bind to the oleic acid affinity column. This suggests that in the proenzyme the hydrophobic sites responsible for the binding of lipase to the affinity resin are obstructed or in some other manner unavailable for binding of the enzyme to the resin. Whether or not these differences between recombinant prolipase and lipase are important *in vivo* is unknown.

The refolded prolipase was purified only by ion exchange chromatography. The resulting preparations contained prolipase and a small amount of lower molecular weight proteins (Fig. 4, lanes f and g). The specific activities of such preparations ranged from 5000 to 8000 U/mg, comparable to those of purified fungal and recombinant mature lipase. The prolipase preparations were of sufficient purity to allow automated sequence determination of the first 20 amino acid residues. As was observed with recombinant lipase, the *N*-terminal methionine residue was still present. The sequence of the remaining 19 amino acid residues was as predicted from the nucleotide sequence of the cDNA (12).

Comparisons of physical properties of fungal lipase, recombinant lipase and recombinant prolipase. Haas *et al.* (8) have previously determined the influence of pH and temperature on the activity of purified fungal lipase. The lipase was most active at a pH around 8, near its predicted pI of 8.1, and the activity and stability were maximum at 30°C. The same characteristics were observed for the purified recombinant lipase (Fig. 6A–C). Recombinant prolipase was most active at pH 7.5, near its predicted pI

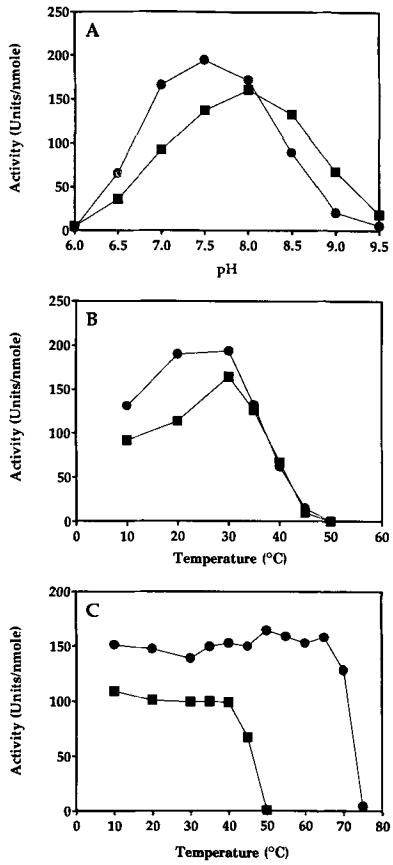

FIG. 6. A. Effect of pH on activity of lipase (—■—) and prolipase (—●—). Samples were assayed titrametrically as described in Materials and Methods. Activities were calculated based on the amount of protein used per assay and on molecular weights of 29,500 for lipase and 39,500 for prolipase. B. Effect of reaction temperature on activity of recombinant lipase and prolipase. Samples were assayed titrametrically in thermostatted reaction vessels as described in Materials and Methods. Activities were calculated as described for panel A. C. Thermal stability of lipase and prolipase. The enzyme samples were incubated at the indicated temperatures for 15 min and assayed at 25°C.

of 7.2 (Fig. 6A). The activity of prolipase was also maximum at 30°C (Fig. 6B). Thus, in their active conformation in the presence of substrate, both prolipase and lipase are equally susceptible to heat-induced denaturation. However, in the absence of substrate, prolipase, but not mature lipase, withstood exposure to temperatures as high as 70°C (Fig. 6C). Evidently, in the absence of an interface, the propeptide delays the onset of denaturation or promotes rapid renaturation of heat-exposed enzyme.

The similarities in the physical characteristics and the virtually identical specific activities suggest that the *R. delemar* lipase produced by *E. coli* and the lipase produced by the fungus could be of equivalent use for most applications. The yield of recombinant lipase after refolding and purification is about one-hundred times higher than that achieved by purification of the enzyme from fungal cultures. Thus a small culture provides sufficient enzyme material for most laboratory applications such as crystallography studies. It should be possible to increase the yield further simply by growing the recombinant *E. coli* in fermentors instead of the shaken flasks used in this study. Also, as our knowledge of protein refolding and appropriate refolding techniques advances, further yield increases should be possible.

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CDPcholine:1,2-Diacylglycerol Cholinephosphotransferase from Rat Liver Microsomes. I. Solubilization and Characterization of the Partially Purified Enzyme and the Possible Existence of an Endogenous Inhibitor

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The solubilization and partial purification of cholinephosphotransferase (CDPcholine:1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2) from rat liver microsomes were examined in the presence of ionic (sodium deoxycholate), nonionic (Triton X-100, *n*-octylglycoside), or zwitter ionic (CHAPS) detergents. Among the four detergents tested, only sodium deoxycholate was found to be an efficient solubilizer of cholinephosphotransferase activity from microsomal membranes, whereas the other three detergents caused irreversible inactivation of the enzyme at the solubilization step. Addition of phospholipids at the solubilization step, or after solubilization of the membrane proteins, could not preserve or reconstitute activity to any extent. The sodium deoxycholate-solubilized activity was partially purified by gel permeation chromatography (Superose 12HR). The partially purified preparation appeared to consist of a large aggregate containing phospholipids; further dissociation of the protein-phospholipid complex caused complete inactivation of the enzyme. The partially purified cholinephosphotransferase showed a specific activity of 100–130 nmol/min/mg protein, which is the highest activity reported to date from any tissue source; this amounts to a 4-fold enrichment of cholinephosphotransferase activity from the original KCl-washed rat liver microsomes. Ethanolaminephosphotransferase (CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase, EC 2.7.8.1) activity was copurified and 6-fold enriched with a total recovery of 60%. During the purification of cholinephosphotransferase activity, a putative endogenous inhibitor of cholinephosphotransferase was also solubilized and was isolated from the microsomal membranes. This heat-labile, nondialyzable inhibitor was shown to act specifically on cholinephosphotransferase and not on ethanolaminephosphotransferase. Further characterization of the inhibitory activity revealed that it may act at the binding step of the cholinephosphotransferase to its lipid substrate, diacylglycerol.

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Phosphatidylcholine and phosphatidylethanolamine are the two major membrane phospholipids in animal cells; together they typically constitute more than 80% of total phospholipids in most biomembranes. Beyond their apparent structural roles, both classes of phospholipid also appear to be involved in the modulation of certain membrane-bound enzymes (1). More recently, the participation of these phospholipids, and particularly of phosphatidylcholine, in signal transduction has also been implicated (2–6).

In most mammalian tissues, the CDPcholine (CDP ethanolamine) pathway is the major biosynthetic route for the formation of phosphatidylcholine (phosphatidylethanolamine). Either pathway involves three enzymatic steps *i.e.*, choline(ethanolamine) kinase, phosphocholine(phosphoethanolamine) cytidylyltransferase, and choline(ethanolamine)-phosphotransferase. In the past few years, enzymes of the first two steps have been purified from mammalian sources to apparent homogeneity. Choline kinase has been purifed from rat kidney (7), liver (8) and brain (9) cytosols and proven to be the same enzyme as ethanolamine kinase in these and other rat tissues (8,10). Phosphocholine cytidylyltransferase has been purified from rat liver cytosol (11,12), and this enzyme is distinct from the corresponding phosphoethanolamine cytidylyltransferase, which also had been purifed earlier from rat liver cytosol (13). The last two enzymes, cholinephosphotransferase (CPT) and ethanolaminephosphotransferase (EPT), which have been shown to be tightly membrane-bound, are so-called integral membrane proteins, and still have not been purified. These enzymes need to be purified to arrive at a better understanding of the molecular mechanisms involved in the regulation of phosphatidylcholine and/or phosphatidylethanolamine biosynthesis in animal cells.

A number of attempts have been made to solubilize CPT from several membrane sources by use of various detergents (14–16), but further efforts to purify the enzyme have not been successful mainly because of insufficient information about the nature of the enzyme and its susceptibility to detergents. EPT, on the other hand, was shown to be relatively stable in the presence of certain nonionic detergents; thus Roberti *et al.* (17) have recently succeeded in the 1200-fold purification of the enzyme upon solubilization from rat brain microsomes with Triton X-100 (TX-100), although their final preparation still showed several protein bands in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

In the present paper, we report (i) the selection of an effective detergent for solubilization of CPT from rat liver microsomes in intact form, (ii) the results of the partial purification of both the choline- and ethanolaminephosphotransferases, (iii) some characteristics of the solubilized, partially purified transferases and (iv) the possible existence of an endogenous inhibitor of CPT activity.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; CPT, cholinephosphotransferase; DOC, sodium deoxycholate; EGTA, ethyleneglycol-*bis*(β-aminoethylether)*N,N,N',N'*-tetraacetic acid; EPT, ethanolaminephosphotransferase; *n*-OG, *n*-octyl-β-D-glucopyranoside; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TX-100, Triton X-100.

MATERIALS AND METHODS

Chemicals. CDP[*methyl*-^{14}C]choline (ammonium salt) and CDP[1,2-^{14}C]ethanolamine were obtained from Amersham Japan Ltd. (Tokyo, Japan) and ICN Biochemicals Inc. (Costa Mesa, CA), respectively. The detergents used were sodium deoxycholate (DOC) and *n*-octyl-β-D-glucopyranoside (*n*-OG) from Sigma (St. Louis, MO), TX-100 from Nakalai Tesque (Kyoto, Japan) and Tween-20 and CHAPS from Bio-Rad (Richmond, CA). All other reagents were of analytical grade. 1,2-Diacylglycerol was prepared from egg yolk phosphatidylcholine by phospholipase C (*Clostridium welchii*) treatment and was purified by thin-layer chromatography (TLC) as described previously (18). The purified 1,2-diacylglycerol was dissolved in $CHCl_3$ at a concentration of 12 mg/mL and stored at −20°C until use.

Preparation of KCl-washed microsomes. Wistar rats of either sex, weighing 200–250 g, were killed by decapitation after overnight fasting. The livers were thoroughly rinsed with ice-cold saline and homogenized in 4 vol of 0.25 M sucrose, 3 mM Tris/HCl (pH 7.5) and 0.1 mM ethylenediaminetetraacetic acid using a Teflon-pestle glass homogenizer. The homogenates were centrifuged at 24,000 × *g* for 10 min, and the resulting supernatant was recentrifuged at 54,000 × *g* for 60 min. The pellet (microsomal fraction) was suspended in approximately 10 vol of 0.25 M sucrose, 10 mM Tris/HCl (pH 8.0), 1 mM histidine, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Buffer A), containing 0.6 M KCl, and stirred for 20 min at 0°C. The suspension was then centrifuged at 200,000 × *g* for 30 min. The resulting pellet (KCl-washed microsomes) was re-homogenized in a small amount of Buffer A (approximately 30 mg microsomal protein/mL) and stored at −20°C until use. Both choline- and ethanolaminephosphotransferase activities were found to be quite stable at this stage even after several thaw-freeze cycles.

Solubilization of CPT activity from KCl-washed microsomal membranes. Four typical detergents commonly used for solubilizing membrane proteins, TX-100, *n*-OG, CHAPS and DOC, were examined to check their efficiency in solubilizing CPT activity. The KCl-washed microsomal fraction (10 mg protein/mL) was incubated for 20 min to 21 h at 4°C in 0.25 M sucrose, 50 mM Tris/HCl (pH ranges of 6.0 to 9.0), 0.8 mM CDPcholine, 1 mM histidine, 0.3 mM PMSF, plus various amounts of the detergents (from 0.05 to 5.0 mg/mg microsomal protein). Detergent concentrations in the present investigation were measured in weight ratios of total detergent to membrane protein following the suggestion that this may be the best way to define the solubilizing power of a detergent (15,19). When DOC was used as solubilizing agent, 1 M KCl was added to the solubilization buffer. The mixture was centrifuged at 200,000 × *g* for 30 min, and the resulting supernatant was dialyzed overnight against 20% glycerol, 20 mM Tris/HCl (pH 8.5), 1 mM dithiothreitol (DTT), 0.5 mM ethyleneglycol-*bis*(aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA) and 0.3 mM PMSF (Buffer B) and then centrifuged at 200,000 × *g* for 30 min. After the pellet was re-homogenized in a small amount of Buffer B, an aliquot of both fractions was assayed for cholinephosphotransferase activity.

The pellet from the initial centrifugation step, *i.e.*, an unsolubilized microsomal membrane fraction, was also rehomogenized in a small amount of Buffer B and assayed to estimate unsolubilized CPT activity. For TX-100-solubilized preparations, Bio-beads SM-2 (Bio-Rad) was used to adsorb the detergent (15) from the solubilized sample after overnight dialysis against Buffer B.

Large-scale solubilization and gel permeation chromatography. KCl-washed rat liver microsomes (10 mg/mL) were solubilized in 0.25 M sucrose, 50 mM Tris/HCl (pH 7.5), 0.8 mM CDPcholine, 1 mM histidine, and 0.3 mM PMSF, containing 6 mM (0.25%) DOC and 1 M KCl, with moderate shaking for 20 min at 4°C; then the mixture was centrifuged at 200,000 × *g* for 30 min. In some experiments, 1,2-diacylglycerol (1.25 mg/mL), leupeptin (10 μg/mL) and aprotinin (10 μg/mL) were also included in the solubilization buffer. The high-speed supernatant was passed through a membrane filter (Millex-GS, 0.22 μm, from Japan Millipore Ltd. Tokyo, Japan), then the total filtrate was dialyzed overnight against Buffer B containing 2.4 mM (0.1%) DOC. The preparation at this stage could be stored at −20°C for at least 4 wk without loss of CPT activity. The solubilized preparation (0.5 mL each) was next applied onto a Superose 12HR column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with Buffer B containing 2.4 mM DOC and 0.15 M KCl at a flow rate of 0.3 mL/min. Fractions of 0.6 mL each were collected and assayed for both choline- and ethanolaminephosphotransferase activities. An aliquot of the fractions was also used for protein assay by the Lowry method (20) and for measuring lipid phosphorus after perchloric acid digestion (21). The fractions with high CPT activity were pooled and dialyzed for 16 h against Buffer B, then centrifuged at 200,000 × *g* for 30 min. The activity was recovered quantitatively in the high-speed pellet fraction, and the pellet was resuspended in a small amount of Buffer B by brief homogenization. At this stage the enzyme activity was stable when stored at −20°C.

Enzyme assay. CPT activity was assayed essentially as described before (18). The typical incubation mixture contained 75 mM Tris/HCl (pH 8.5), 10 mM $MgCl_2$, 5 mM EGTA, 3 mM DTT, 2 mM 1,2-di-acylglycerol (in final 0.02% Tween-20 emulsion), 0.5 mM CDP[*methyl*-^{14}C]-choline (specific activity, 0.1 Ci/mol) and an aliquot of enzyme preparation in a final volume of 0.2 mL. Incubations were started by addition of enzyme and carried out for 20 min at 37°C. The reaction was stopped by addition of 2 mL of $CHCl_3$/MeOH (2:1, vol/vol), followed by extraction of the reaction product, phosphatidylcholine, into $CHCl_3$. Blanks were usually done by stopping the reaction immediately after the addition of the enzyme. When appropriate, the activity without exogenous 1,2-diacylglycyerol (0.02% Tween-20 only) was subtracted as endogenous activity. The $CHCl_3$ layer was washed first with 2.5 mM cold CDPcholine (in 50% MeOH solution), then twice with 50% MeOH. The washed $CHCl_3$ layer was transferred into a liquid scintillation vial, dried under N_2 and counted for radioactivity in 10 mL ACS-II (Amersham) scintillation cocktail. The amount of phosphatidylcholine formed during the incubation was estimated from the specific radioactivity of CDP-[*methyl*-^{14}C]choline. The reaction was almost linear for 40 min (50 μg protein) and up to 100 μg protein (20-min

incubation) when the KCl-washed microsomal fraction was used as the enzyme source.

EPT activity was assayed in exactly the same manner as was CPT except that 10 mM $MgCl_2$ was replaced by 10 mM $MnCl_2$, and 0.5 mM CDP[1,2-^{14}C]ethanolamine (specific activity, 0.25 Ci/mol) was used instead for CDP-[*methyl*-^{14}C]choline. The specific activity of EPT in KCl-washed microsomes ranged around 4–5 nmol/min/mg protein; this value was one-fifth to one-seventh of that of CPT in the same enzyme preparation. The 1,2-diacylglycerol/Tween-20 emulsion (20 mM diacylglycerol) was prepared as follows: twelve milligram (20 μmoles) of dried 1,2-diacylglycerol was added in 1 mL of 0.2% Tween-20 solution and sonicated with a probe-type sonicator until no floating material remained. Usually three irradiation cycles of 30 s each were necessary (at 0°C). The emulsion could be stored at −20°C, but resonication just before use was necessary. For some experiments, 1,2-diacylglycerol (final 1.25 mM) was emulsified in 5 mM DOC and 0.25% Tween-20 mixtures according to the method of Kanoh and Ohno (14,22), and enzyme activities were compared for the two substrates in their different physical states.

In another experiment with the partially purified enzyme, total lipids from rat liver microsomes (extracted by the Folch method; ref. 23) were added to the incubation mixture as a 10 mM Tris/HCl (pH 7.5) dispersion to arrive at a final phospholipid concentration of 0.5 mM (estimated by lipid phosphorus).

Analysis of water-soluble products. After the reaction of CPT was stopped by the addition of 1.5 mL $CHCL_3$/MeOH (2:1, vol/vol), an aliquot of the resulting aqueous layer was applied to a TLC plate (Kiesel Gel 60 from Merck, Darmstadt, Germany) together with small amounts of carrier CDP—choline, phosphocholine and choline. The developing solvent system used was MeOH/0.6% NaCl/conc. NH_4OH (10:10:1, by vol). Fractions corresponding to each of the metabolites were detected by exposure to iodine vapor, scraped from the plate, transferred to a scintillation vial and counted using 1 mL H_2O plus 10 mL ACS-II.

RESULTS

Effect of detergents on CPT activity in rat liver microsomes. Before comparison of the solubilization efficiencies of the four detergents for CPT from liver microsomal membranes, the effect of the detergents on CPT activity was examined as a function of detergent concentration. As shown in Figure 1, all the detergents examined were found to inhibit CPT activity in a dose-dependent manner. When the dose was plotted as mg detergent per mg microsomal protein, the inhibition by TX-100 was most striking, followed by DOC, CHAPS and *n*-OG. The observations essentially agree with those reported by Cornell and MacLennan (15), who compared the effect of six different detergents on CPT activity from rabbit sarcoplasmic reticulum. The order of enzyme inhibition observed in the present investigation was essentially parallel to the detergents' capability to solubilize membranes (15), indicating that the inhibition of CPT activity was most likely due to the disruption of the membrane structure. Or conversely, the activity of CPT appeared to be very much dependent on its lipid environment, as was already suggested by Cornell and MacLennan (15). The possibility that the presence of detergent in the assay could also prevent the utilization of exogenous 1,2-diacylglycerol by the enzyme cannot be entirely ruled out.

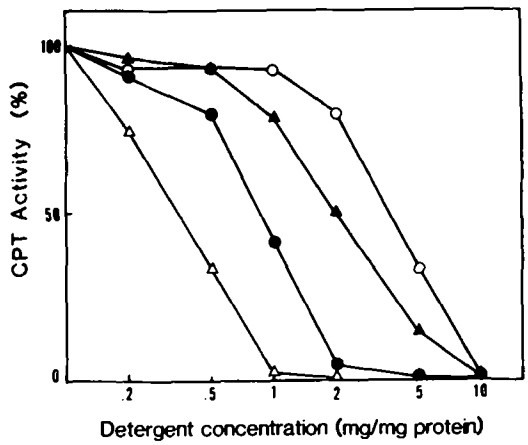

FIG. 1. Detergent sensitivity of cholinephosphotransferase activity from rat liver microsomes. Detergents were added directly to the cholinephosphotransferase assay mixtures at the indicated concentrations. The reaction was started with the addition of KCl-washed microsomes (50 μg protein) and continued for 20 min at 37°C. The specific activity in the absence of detergents ranged 30–35 nmol/min/mg protein. Data are averages of two separate experiments. △, TX-100; ●, DOC; ▲, CHAPS; ○, *n*-OG. CPT, cholinephosphotransferase.

Our results therefore indicate that, after solubilization of CPT from microsomal membranes by the detergents, the detergents must be removed from the solubilized preparation before enzyme activity is assayed.

In contrast to several reports which showed an activation of CPT by a low concentration of either TX-100 or DOC, we observed no stimulation of enzyme activity by these detergents. This may be due to differences in the physical state of the exogenously added lipid substrate. We used 1,2-diacylglycerol/Tween-20 emulsion, whereas phospholipid mixed micelles or ethanol dispersions were used in the earlier experiments (14, 15,24).

Solubilization of CPT from microsomal membranes. The solubilizing efficiency of the four detergents for CPT activity from KCl-washed rat liver microsomes was compared as a function of detergent concentration (from 0.05 to 5 mg/mg protein), incubation time (from 20 min to 21 h) and pH (from 6 to 9). Typical results are shown in Figure 2, in which CPT activities that were recovered in the solubilized fraction after the incubation with the detergents at various concentrations are compared. Both the total protein solubilized from the microsomal membranes and the unsolubilized CPT activity are given also as percentages of the values found in the initial microsomal membranes. The results suggest that, among the four detergents examined, only DOC effectively solubilized the microsomal CPT in active form. Other nonionic (TX-100 and *n*-OG) and zwitter ionic (CHAPS) detergents were found to cause irreversible inactivation

of the enzyme, which resulted in recovery of only 1 to 3% of the initial activity in the solubilized fraction. The inclusion of phospholipid dispersions (0.5 mM, isolated from rat liver microsomes) in the medium before or after the incubation with detergents did not significantly improve the recovery of enzyme activity.

Our result also showed that almost all of the CPT activity solubilized by DOC was recovered in the high-speed pellet when the detergent was removed by overnight dialysis against Buffer B. Prolonged incubation from 20 min to 2 h, 4 h or 21 h at pH 8.0 did not increase but actually did diminish the recovery of enzyme activity solubilized by the detergents (data not shown). Also a shift of pH between 6 and 9 did not significantly affect the solubilizing efficiency of DOC for CPT activity, but somewhat higher recoveries of both the enzyme activity and the protein were obtained at pH 9.0 with either CHAPS or TX-100; *i.e.*, 1% at pH 8.0 *vs.* 3.5% at pH 9.0 for CHAPS, and 2.5% at pH 8.0 *vs.* 6% at pH 9.0 for TX-100 (data not shown).

Cornell and MacLennan (15) had reported that inclusion of diacylglycerol at the solubilization step of the isolation of CPT from rabbit sarcoplasmic reticulum stabilized and partly protected enzyme activity from irreversible inhibition by detergents. We checked the effect of addition of diacylglycerol to the solubilizing medium with DOC and found that, if 0.8 mM CDPcholine, the other substrate of CPT, was included in the medium, there was no further stabilization or protection of enzyme activity by diacylglycerol. In addition, as mentioned in Materials and Methods, inclusion of diacylglycerol led to considerably higher endogenous activity values which could make it difficult to correctly estimate the enzyme activity during the subsequent enzyme purification. Thus, we decided not to include diacylglycerol at the solubilization step using DOC.

Partial purification of CPT. As DOC was the most effective agent for solubilizing CPT from rat liver microsomes, we next proceeded to purify the enzyme activity from the DOC-solubilized preparation by Superose 12HR column chromatography using a Pharmacia FPLC system. Figure 3 shows a typical elution pattern of CPT activity from the column together with the protein and lipid phosphorus values determined on the same run. The elution of EPT activity is also plotted in Figure 3. The results clearly show that both choline- and ethanolaminephosphotransferase activities are recovered in the fractions close to the void volume, suggesting that most of the activity is associated with large aggregates which still contain considerable amounts of phospholipids. Well-dissociated fractions of smaller molecular size were found not to exhibit any phosphotransferase activity with either CDPcholine or CDP—ethanolamine. CPT activity could be purified by gel permeation chromatography about four-fold with a recovery of approximately 40% when compared to the original microsomal activity (Table 1). EPT activity was purified 6-fold with 60% recovery, indicating that the latter activity remained relatively stable in the course of the solubilization-purification process. A number of reports had previously indicated that EPT, which is an enzyme distinct from CPT (25-28), is much more stable than CPT in the presence of various detergents, including DOC (14,29-31).

The SDS gel (Fig. 4) shows that the polypeptide pattern of the fraction with high choline- or ethanolaminephosphotransferase activity after gel permeation chromatography (lane C) was quite different from that of either the microsomes (lane A) or the DOC-solubilized fraction (lane B), indicating that the large aggregates in this fraction could not simply be fragments of microsomal membranes; however, it could be a complex of particular species of microsomal proteins which appear not easily dissociable in the presence of DOC.

Further attempts to purify CPT with a DEAE-Sepharose or hydroxyapatite column in the presence of DOC were not successful because of the extremely low recovery of enzyme activity.

It has been reported that Mn^{2+} is a preferable cation in the EPT reaction in most membrane systems (14,22,24, 31-33) whereas in certain mammalian systems Mg^{2+} is known to be more active in CPT reaction (14,22,31,34). Kanoh and Ohno (14) reported that, in rat liver microsomes, there might be two components contributing to

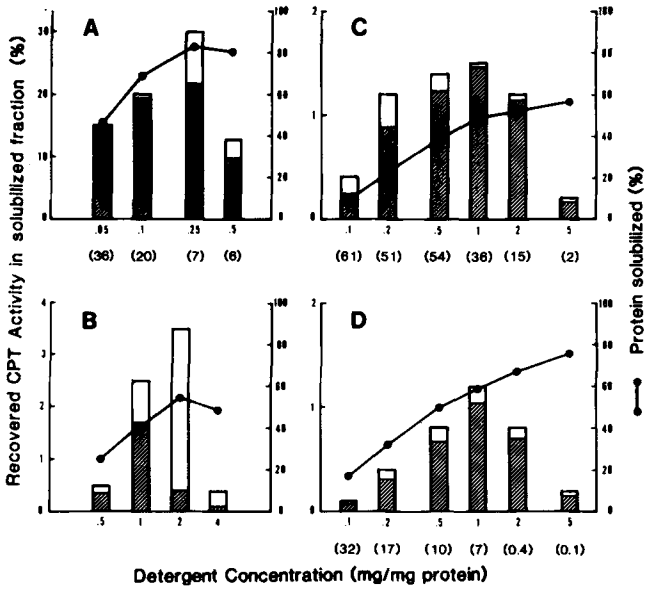

FIG. 2. Solubilization of cholinephosphotransferase activity from rat liver microsomes by detergents. The KCl-washed microsomes (10 mg protein/mL) were incubated for 20 min at 4°C in 0.25 M sucrose, 50 mM Tris/HCl (pH 8.0), 0.8 mM CDPcholine, 1 mM histidine, 0.3 mM PMSF and the indicated amounts of detergents in a final volume of 1.0 mL. 1 M KCl was also included in the case of DOC-solubilization, which resulted in a 25% increase in the total solubilized activity. The addition of KCl did not improve the recovery by other detergents. The contents were centrifuged at 200,000 × *g* for 30 min, and the resulting supernatant was dialyzed overnight against Buffer B, then recentrifuged at 200,000 × *g* for 30 min. The Triton-100-solubilized preparation was treated with Bio-beads SM-2 to adsorb the detergent (ref. 15) before the second centrifugation. Each column represents the total solubilized activity (as percentage of the initial microsomal activity) determined after removal of detergent, and the hatched portion represents the activity recovered in the pellet after the second high-speed centrifugation. The values in parentheses at the bottom represent the activity recovered in unsolubilized microsomal membrane remnants (as percentages of the initial microsomal activities). A, sodium deoxypanesulfonate; B, *n*-octyl-β-D-glucopyranoside; C, CHAPS; D, Triton-100. CPT, cholinephosphotransferase.

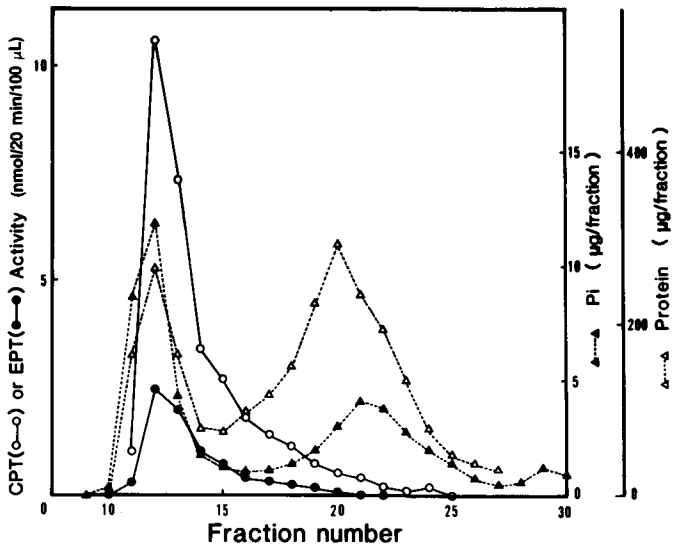

FIG. 3. Typical elution pattern of cholinephosphotransferase and ethanolaminephosphotransferase activities through a Superose 12HR column. An aliquot of the DOC-solubilized fraction (usually 0.5 mL, approx. 4 mg protein) was applied onto the column (1 × 30 cm) and eluted with Buffer B containing 2.4 mM (0.1%) DOC and 0.15 M KCl as described in Materials and Methods. Fractions of 0.6 mL each were collected and assayed for both choline (○)- and ethanolamine (●) phosphotransferase activities as well as for protein (△) and lipid phosphorus (▲) contents. Abbreviations used are: CPT, cholinephosphotransferase; EPT, ethanolaminephosphotransferase; DOC, sodium deoxycholate.

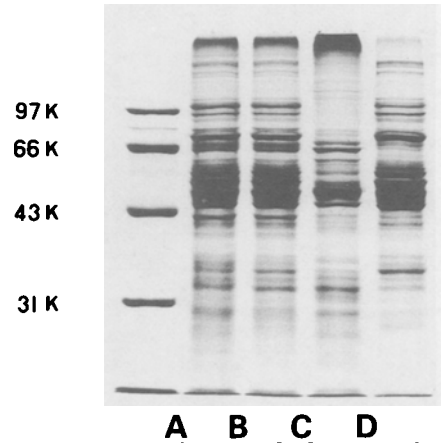

FIG. 4. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis of rat liver choline(ethanolamine)phosphotransferase preparations. Each lane contained 50 μg protein from KCl-washed microsomes (A), sodium deoxycholate-solubilized fraction (B), partially purified enzyme preparation (C) and inhibitor Fraction 17 (D) obtained by Superose 12HR gel filtration. The left side lane shows molecular weight standards; rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (22 kDa, bottom). A 10% SDS-polyacryalmide gel was run according to the method of Laemmli (ref. 40) and the protein was stained with Coomassie Brilliant Blue R-250.

CPT activity, one being Mg^{2+}-requiring and the other being Mn^{2+}-requiring; both could be partly separated from each other by sucrose density gradient centrifugation in the presence of 0.5% TX-100. We could not reconcile our results (Fig. 5) with those reported by Kanoh and Ohno (14,22) as well as others (35,36) working with rat tissue enzymes. Our preparations of either partially purified or microsomal phosphotransferases required Mg^{2+} rather than Mn^{2+}, particularly at physiological concentrations (less than 5 mM). when compared to the CPT, Mn^{2+} could more effectively replace Mg^{2+} in the EPT reaction but was never preferred over Mg^{2+} in either the microsomal preparation or the partially purified enzyme. These differences in cation requirements may partly be due to differences in the assay system, where a 0.02% Tween-20 emulsion of diacylglycerol was used in the present investigation. Alternatively, more than one species of both choline- or ethanolaminephosphotransferase could exist in rat tissues.

Possible existence of a CPT inhibitor protein in rat liver microsomes. During the process of CPT purification by gel permeation chromatography, we found that the DOC-solubilized preparation (after removal of DOC by overnight dialysis against Buffer B) gave a nonlinear, satur-

TABLE 1

Partial Purification of Cholinephosphotransferase (CPT) and Ethanolaminephosphotransferase (EPT) Activities from Rat Liver Microsomes[a]

	CPT		EPT		
	S.A.[b]	T.A.[c]	S.A.[b]	T.A.[c]	CPT/EPT
	nmol/min/mg	nmol/min	nmol/min/mg	nmol/min	
KCl-washed microsomes	32	8463	4.5	1193	7.1
DOC-solubilized	23	5132	4.3	972	5.3
Superose 12HR	129	3251	28	705	4.6
Purification (fold)	4.0	—	6.2	—	—
Recovery (%)	—	38.4	—	59.1	—

[a]Purification was started with 265 mg KCl-washed microsomal protein. The activities in DOC-solubilized and Superose 12HR fractions were determined after the removal of DOC by overnight dialysis against Buffer B. DOC, sodium deoxycholate.
[b]S.A., specific activity.
[c]T.A., total activity.

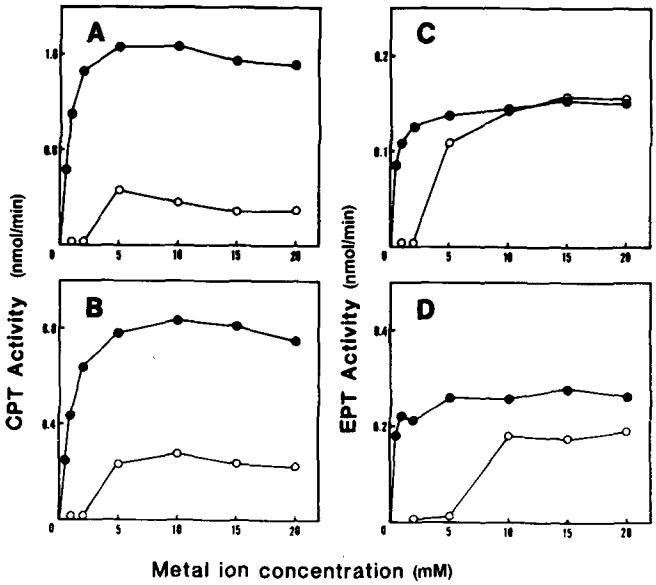

FIG. 5. Comparison of divalent cation dependency between cholinephosphotransferase and ethanolaminephosphotransferase in both microsomal and partially purified enzyme preparations. Each incubation contained 50 μg (microsomes) or 7 μg protein (partially purified enzyme) in a final volume of 0.2 mL. Other experimental details are those described in Materials and Methods. A and C, microsomal activity; B and D, partially purified enzyme activity. ●, activity with Mg^{2+}; ○, activity with Mn^{2+}. Abbreviations used are: CPT, cholinephosphotransferase; EPT, ethanolaminephosphotransferase.

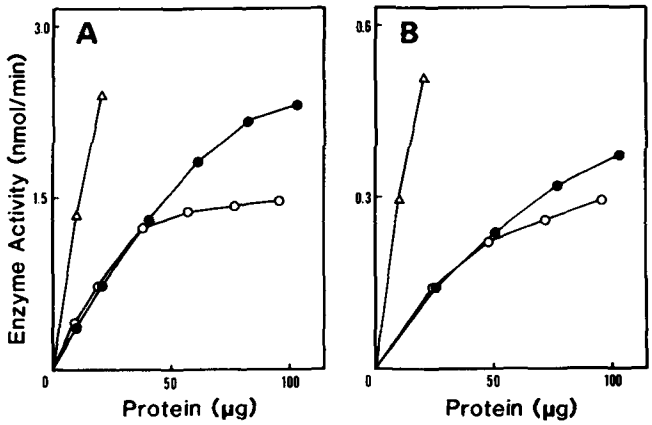

FIG. 6. Linearity of cholinephosphotransferase (A) and ethanolaminephosphotransferase (B) activities with microsomes, sodium deoxycholate (DOC)-solubilized, and partially purified enzyme preparations for increasing protein concentrations. The activity was determined after removal of DOC by overnight dialysis both for the DOC-solubilized and partially purified enzyme preparations. ●, microsomes; ○, DOC-solubilized enzyme; △, partially purified enzyme obtained with a Superose 12HR column.

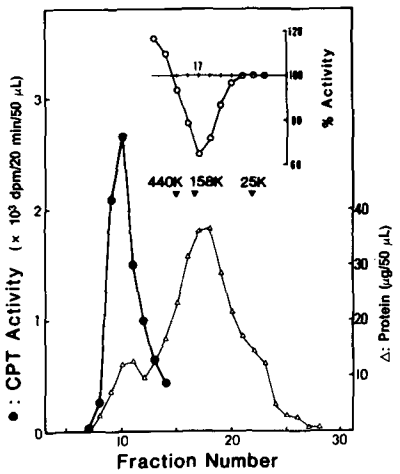

FIG. 7. The elution pattern of inhibitor activity obtained with a Superose 12HR gel filtration column. The fractions eluted after the peak of cholinephosphotransferase activity were dialyzed for 24 h against Buffer B and a 35-μL portion of each fraction was added to the assay mixture which contained the partially purified enzyme preparation (10 μg protein). The inhibitor activity was plotted (inset) as percent of the remaining cholinephosphotransferase activity, in which Fraction 17 showed the highest inhibition. Arrows indicate the elution peaks of the molecular weight standards from Pharmacia; ferritin (440 kDa, from horse spleen), aldolase (158 kDa, from rabbit muscle) and chymotrypsinogen A (25 kDa, from bovine pancreas). CPT, cholinephosphotransferase.

able curve when the enzyme activity was plotted as a function of protein content, as shown in Figure 6. This nonlinearity was not as pronounced when microsomal membranes were used as the enzyme source and was never detected with the partially purified CPT preparation from the Superose 12HR column. The effect was less pronounced when the relationship was plotted for EPT activity (Fig. 6B) using the same DOC-solubilized enzyme preparation.

It appears that the inhibition of CPT activity observed at higher protein concentrations may have been caused by the presence of an endogenous inhibitor that was solubilized together with the CPT by DOC and removed from the active enzyme fraction in the subsequent gel permeation step. In order to characterize the nature of the putative CPT inhibitor, samples of the partially purified enzyme preparation were mixed with aliquots of the fractions (after 24 h dialysis against Buffer B) eluted from the gel following the CPT activity peak and the activities were assayed. It is clear from Figure 7 that Fraction 17 contained the highest inhibitory activity. This fraction inhibited CPT activity in a dose-dependent manner (Fig. 8), and the inhibition appeared to be specific for CPT because the fraction did not significantly inhibit EPT activity. The inhibitory activity of Fraction 17 was completely abolished when the fraction was pre-heated for 2 min in a boiling water bath (data not shown).

The SDS-PAGE pattern of Fraction 17 (Fig. 4, lane D) was found to be quite different from that of the partially purified enzyme fraction (Fig. 4, lane C).

The inhibition of CPT was further characterized by measuring reaction kinetics. The partially purified preparation gave a saturation activity curve *vs.* CDPcholine and an apparent Km value for CDPcholine of 58 μM. This value became smaller in the presence of the inhibitor, and the inhibition appeared to be uncompetitive *vs.* CDPcholine (data not shown). An exact

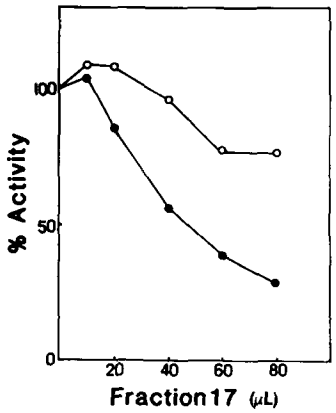

FIG. 8. The inhibition of cholinephosphotransferase and ethanolaminephosphotransferase activities by Fraction 17. An aliquot of the partially purified enzyme preparation (20 μg protein) was added by various amounts of Fraction 17 (DOC had been removed by 24-h dialysis against Buffer B); then the mixture was assayed for both choline- and ethanolaminephosphotransferase activities. The protein content of Fraction 17 was approx. 37 μg/50 μL; ●, cholinephosphotransferase; ○, ethanolaminephosphotransferase.

Km value for diacylglycerol could not be obtained because our assay system using exogenous diacylglycerol in Tween-20 emulsion gave a sigmoidal activity curve against diacylglycerol.

Next, the water-soluble choline metabolites of the CPT assay were analyzed in the absence or presence of the inhibitor protein to test the possibility that the apparent inhibition might be due to [^{14}C]CDPcholine hydrolytic activity. Although some hydrolyzing activity toward CDPcholine (or phosphatidylcholine) was observed in both the enzyme and inhibitor fractions, it seemed unlikely that the inhibition of CPT activity by Fraction 17 occurred at the level of CDPcholine hydrolysis, since more than 80% of [^{14}C]CDPcholine was recovered in its intact form after incubation in either the presence or absence of the inhibitor fraction (data not shown).

Finally, the inhibitory activity of Fraction 17 was tested in a different CPT assay system to check the possibility of whether the inhibitor might act on the diacylglycerol dispersion by rendering its physical state ineffective as substrate. We compared our assay method to that of Kanoh and Ohno (14,22), in which diacylglycerol (0.5 mM) was added in mixed micelles of phospholipids (0.5 mM)/Tween-20 (0.1%)/DOC (2 mM). While the specific CPT activity measured by this method was considerably lower (12–13 nmol/min/mg protein without phospholipids and 15–18 nmol/min/mg protein with 0.5 mM phospholipids) than when measured by ours (80–130 nmol/min/mg protein with or without added phospholipids), the activity measured essentially was not affected by the addition of the inhibitor fraction to the assay system (data not shown).

Thus, one could speculate that the putative endogenous inhibitor may act not only on the enzyme substrate (CDPcholine) complex, but also on the diacylglycerol/Tween-20 emulsion and may change its physical state in a way so it cannot be utilized effectively by the enzyme.

DISCUSSION

Recently, O and Choy (16) reported the partial purification of CPT solubilized from hamster liver microsomes with 3% Triton QS-15 using a DEAE-Sepharose column followed by Sepharose 6B gel filtration. The purified preparation had a specific activity of 3.68 nmol/min/mg protein; this value was approximately seven times that of the original microsomal preparation (0.5 nmol/min/mg protein). We applied this method to the isolation of CPT from rat liver microsomes and found that the enzyme solubilized with Triton QS-15 was extremely unstable when kept at 0°C in the presence of 1–3% Triton QS-15, and particularly in dilution (less than 1 mg protein/mL). In addition, the enzyme could not sustain most of its activity during a thaw freeze cycle.

Thus, in our system, DOC appeared to be a more effective detergent for solubilizing CPT. Subsequent purification of the DOC-solubilized enzyme through a Superose 12HR column in the presence of 0.1% DOC resulted in a 4-fold enrichment of activity with a 40% recovery in respect to the original microsomal activity. The result is similar to that reported by Kanoh and Ohno (14,22), who also achieved a 4-fold purification of CPT activity through differential pH treatment of rat liver microsomes in the presence of 16–20 mM DOC. The specific activity reported for their partially purified enzyme was 16–20 nmol/min/mg protein, which was less than the microsomal activity measured in our study (approx. 30 nmol/min/mg protein). This difference could be explained by the fact that we started the experiment with 0.6 M KCl-washed microsomes and that a considerable portion of the loosely attached, peripheral proteins had already been removed from the microsomal fraction. Furthermore, the specific activity measured using exogenously added diacylglycerol in Tween-20 emulsion, as we used in this study, was found to be much higher than that reported by Kanoh and Ohno (14,22) who used diacylglycerol in mixed micelles (phospholipid/Tween-20/DOC).

The partially purified enzyme obtained by gel filtration appeared to consist of a relatively large aggregate of particular microsomal proteins and phospholipids as it eluted near the void volume as a fairly milky dispersion. Further attempts to replace phospholipid by detergent were not successful. Thus, it appears that dissociation of the enzyme-phospholipid complex causes complete and irreversible inactivation of CPT activity. The results of our purification study thus would indicate that it may be extremely difficult to purify CPT from microsomal membranes by classical methods, as was also emphasized by Cornell in her recent review (37). Finally, it may be more effective to use affinity chromatography in the presence of DOC, in which CDPcholine would be coupled to either an epoxy-activated or a CH-activated Sepharose; such experiments are in progress in our laboratory.

The possible existence of an endogenous CPT inhibitor in rat liver microsomes was indicated in the present study. To our knowledge, this is the first description of such an inhibitor, which presumably is a protein, of the CPT reaction. The possible participation of a cytosolic phosphatidylcholine transfer protein in the regulation of CPT

activity in rat liver microsomes has been reported recently (38). Although the exact mechanism of the inhibition remains to be determined, the results of the present study suggest that the inhibition most likely occurs at or near the binding site between the enzyme and the lipid substrate. One could, thus, postulate that this inhibitory protein component regulates the availability of diacylglycerol to CPT (but not to EPT) in microsomal membranes. Metabolically, diacylglycerol is located at a branch point of the biosynthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol, and the mechanisms for the regulation of specific lipid pathways from diacylglycerol have not yet been identified (39). The actual involvement of a specific regulatory protein for each of the biosynthetic reaction, as may be suggested by this report, would offer an attractive and plausible mode of regulation.

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Fatty Acid Synthesis from [2-^{14}C]Acetate in Normal and Peroxisome-Deficient (Zellweger) Fibroblasts

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The incorporation of [2-^{14}C]acetate into the lipids of normal and peroxisome-deficient (Zellweger's syndrome) skin fibroblasts was examined. Most of the label was incorporated into triacylglycerol fatty acids in normal as well as Zellweger's syndrome cells. Triacylglycerols and cholesteryl esters in Zellweger's syndrome cells contained increased levels of labelled saturated and monounsaturated very long-chain fatty acids (VLCFA, that is fatty acids with more than 22 carbon atoms), in particular hexacosanoic (26:0) and hexacosaenoic (26:1) acids. As traces of labelled VLCFA with up to 32 carbon atoms were detected in triacylglycerols even in control cells it is probable that these fatty acids are formed naturally during the elongation process. Our data suggest that peroxisomes are involved in the chain shortening of the saturated and monounsaturated VLCFA.
Lipids 28, 97-102 (1993).

It has become increasingly apparent that peroxisomes play an important role in a number of lipid metabolic pathways. While some of these pathways, for example fatty acid β-oxidation (1) and prostaglandin β-oxidation (2), are associated with catabolic processes, a number of biosynthetic pathways have also been reported. Thus, the biosynthesis of the 1-*O*-alkyl glyceryl ether bond is now believed to occur only in peroxisomes because the enzymes catalyzing the first two steps, namely dihydroxyacetonephosphate acyltransferase and alkyldihydroxyacetone phosphate synthase, are concentrated in this organelle (3-5). Similarly, the final step in the synthesis of bile acids, which involves an oxidative cleavage of 27-carbon bile acid precursors, is also thought to occur predominantly in peroxisomes (6).

As part of our ongoing interest in the role of peroxisomes in lipid metabolism, we have been investigating the metabolism of [1-^{14}C]-labelled long-chain and very long-chain (>22 carbon atoms, VLCFA) saturated and polyunsaturated fatty acids in normal cultured skin fibroblasts and fibroblasts from patients with Zellweger syndrome (7,8), a rare autosomal recessive disorder characterized ultrastructurally by a nearly complete deficiency of peroxisomes in all tissues thus far examined (9). We have found that labelled acetate, released from [1-^{14}C]-labelled VLCFA by normal cells in culture, is used for the biosynthesis of other fatty acids, presumably either by *de novo* synthesis or by elongation of existing fatty acids. In contrast, this process is impaired in Zellweger cells (7). While it is likely that the defect in [^{14}C]fatty acid synthesis from [1-^{14}C]VLCFA in Zellweger cells is due to the impaired β-oxidation activity and the concomitant reduction in [1-^{14}C]acetate release, it is not known whether lipid synthesis from acetate is also impaired in peroxisome-deficient cells.

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Abbreviations: BHT, butylated hydroxytoluene; BMEM, basal modified Eagle's medium; VLCFA, very long-chain fatty acids; TLC, thin-layer chromatography.

In this study we have investigated the ability of normal and Zellweger cells to synthesize fatty acids from acetate. Our results indicate that the distribution of labelled acetate in VLCFA is abnormal in peroxisome-deficient cells. These findings confirm the importance of peroxisomal function in lipid metabolism.

MATERIALS AND METHODS

Materials. Reverse phase KC-18 thin-layer plates were purchased from Whatman Inc. (Clifton, NJ), and thin-layer silica gel plates were obtained from E. Merck (Darmstadt, Germany). Basal modified Eagle's medium (BMEM) and fetal calf serum were purchased from Flow Laboratories (North Ryde, N.S.W., Australia). Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) was from Commonwealth Serum Laboratories (Melbourne, Australia). Butylated hydroxytoluene (BHT) and the unlabelled lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO). Bio-Rad protein assay dye reagent concentrate was from Bio-Rad Laboratories (Richmond, CA), and autoradiography film (Hyperfilm-^{3}H) from Amersham Australia Pty Ltd. (North Ryde, Australia). OptiPhase LKB scintillation solvent (HiSafe 3) was purchased from LKB-Produckter AB, (Bromma, Sweden). [2-^{14}C]Acetic acid, sodium salt (specific activity 57 mCi/mmol), [1-^{14}C]palmitic and [1-^{14}C]stearic acids were obtained from DuPont Company (Wilmington, DE). [1-^{14}C]Lignoceric(24:0) and [1-^{14}C]hexacosanoic (26:0) acids were synthesized as described by Singh and Poulos (10). The radiolabelled fatty acids were used as standards for reverse phase thin-layer chromatography (TLC). Unlabelled monoenoic fatty acid standards with 28 to 34 carbons were synthesized as described by Johnson (11).

Patients. Cultured skin fibroblasts from patients with Zellweger's syndrome were generously provided by Dr. L. Sheffield and Dr. G. Thompson (Royal Melbourne Children's Hospital), Dr. C. Oley (Mater Misericordiae Mothers' Hospital, Brisbane) and Dr. J. McGill (Royal Children's Hospital, Brisbane). The diagnosis of Zellweger's syndrome was based on clinical history and examination and confirmed by biochemical and, where possible, ultrastructural investigation (12).

Incubation of [2-^{14}C]acetate with fibroblasts in culture. Cultured skin fibroblasts from normal subjects and from Zellweger's syndrome patients were grown in tissue culture flasks (75 cm^2) in BMEM containing 10% fetal calf serum until confluent. One hundred μL of an ethanolic solution of [2-^{14}C]acetic acid (100 μCi) was added to individual culture flasks, and the flask tops were sealed tightly to prevent the escape of radioactive carbon dioxide into the atmosphere. For pulse labelling, incubations were carried out for 3 d prior to processing of the cells. Pulse-chase experiments were carried out using matched flasks of cells. For pulse-chase experiments the medium was removed after 3 d and replaced with fresh medium (without labelled acetate) and the incubation was continued for a further 7 d, with an additional medium change on day 4 of the chase period.

At the end of the incubation period the medium was removed and the cells were washed with Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) prior to harvesting by trypsinization. The harvested cells were washed a further three times with 10 mL of Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and suspended in 450 μL Dulbecco's phosphate-buffer, Ca^{2+}-free but containing 4 mM magnesium chloride. The cells were disrupted by ultrasonication, and aliquots were removed for protein analysis (50 μL) and liquid scintillation counting (5 μL). Protein estimations were carried out as described by Bradford (13).

Lipid extraction and analysis. Lipids were extracted from the cell suspensions by the Bligh and Dyer method (14). BHT was added at all stages of extraction and analysis to minimize autoxidation of polyunsaturated fatty acids. For some experiments the delipidated cellular residue was treated with 4N HCl for 17 h at 100°C and the liberated fatty acids were extracted into hexane. Aliquots of lipid extracts were streaked as 1.5 cm-bands onto silica gel 60 thin-layer plates (20 × 20 cm) and developed about 7 cm with chloroform/methanol/water (65:25:4, by vol). After air drying, the plates were then run in the same direction in chloroform all the way to the top of the plate. This chromatographic system resolved the major phospholipid and neutral lipid classes but did not permit the separation of triacylglycerols and cholesteryl esters. Separation of these particular lipids was achieved by chromatographing lipid extracts in hexane/diethyl ether/glacial acetic acid (90:10:1, by vol). Tentative identification of the various lipid components was achieved by running appropriate standards. Individual zones were made visible by autoradiography. For some experiments the lipid extracts were saponified by treatment with 20% (wt/vol) potassium hydroxide in ethanol/water (67:33, vol/vol) for 3 h at 80°C prior to chromatography (15).

Lipid hydrolysis and methylation of the liberated fatty acids and purification of the fatty acid methyl esters was carried out as described earlier (7). Separation of the fatty acid methyl esters into saturated, monounsaturated and polyunsaturated fatty acid fractions was achieved by argentation TLC (7). Saturated and monounsaturated fatty acid methyl esters were separated according to carbon chain length by reverse phase TLC as described by Street *et al.* (7). Individual fatty acid zones were detected by autoradiography, scraped from the plate into glass scintillation vials and counted in 8 mL scintillation fluid. Where possible, tentative identification of the various saturated and monounsaturated fatty acids was based on a comparison of their mobilities on argentation and reverse phase TLC with those of authentic commercially available or specially synthesized standards. Unlabelled monounsaturated fatty acid standards (16:1-34:1) were detected on reverse phase TLC plates by exposure to iodine vapor. Labelled saturated fatty acid standards (16:0, 18:0, 24:0 and 26:0) were detected by autoradiography.

RESULTS

Approximately 3-4% of the label from [2-^{14}C]acetate was recovered in normal cells harvested 3 d after the addition of the label. About half of this radioactivity was recovered in the lipid fraction after extraction of the cells. Similar results were obtained with Zellweger's syndrome cells (Table 1). After removal of the radioactivity from the medium, the amount of label in the cellular lipids gradually decreased. After a 7-d chase, around 30 to 50% of the label remained within the lipid fraction in both control and Zellweger cells (Table 1).

TABLE 1

Lipid Associated Radioactivity Recovered in Cell Extracts[a]

	Treatment method (nmol labelled acetate/mg protein)	
	3-d Pulse	Pulse chase
Control		
No. 1	85	27
No. 2	99	22
No. 3	104	31
No. 4	152	45
mean (n=4)	110	31
Zellweger		
No. 1	57	28
No. 2	67	44
No. 3	68	57
No. 4	92	25
mean (n=4)	71	39

[a]Control and Zellweger cells (four separate lines of each with each line taken from a different individual) were pulsed with [2-^{14}C]-acetate or pulse-chased as described in the text. The amount of label recovered in the lipid fraction of each cell line is tabulated.

Analysis of the lipid extracts revealed that the label was incorporated into all of the major phospholipid and neutral lipid species in normal cells. At least 95% of the label was recovered as fatty acid after alkaline hydrolysis, indicating that little was incorporated into the hydrophilic moieties of the various phospholipid molecules and most was associated with esterified fatty acids. Small amounts of label were detected in cholesterol, unesterified fatty acids, dolichol, squalene and ubiquinone. The major neutral lipid species labelled were triacylglycerols with up to 65% of the label appearing in these lipids. Cholesteryl esters were also labelled but the proportion of the total label rarely exceeded 2% in this period. After removal of the radioactivity from the medium, the amount of label in the various lipids, and in particular in the triacylglycerols, decreased markedly. After a 7-d chase, the amount of label in the latter dropped to a value of <5% of the total cellular lipid radioactivity (control mean 1.15, range 0.24-2.8 *vs.* Zellweger mean 1.7, range 0.7-2.5 nmol labelled acetate/mg protein).

An examination of chromatograms of lipid extracts revealed that Zellweger triacylglycerols and cholesteryl esters contained faster migrating (and hence more hydrophobic) molecular species than those observed in control cells indicating that the fatty acid labelling patterns were probably different in Zellweger cells (Fig. 1). Reverse phase TLC of the labelled fatty acids released from triglycerides and cholesteryl esters after the 3-d labeling period confirmed this. Most of the label in triacylglycerols (>75%) was recovered in saturated fatty acids, predominantly palmitic and stearic acids, with a lesser amount in monounsaturated fatty acids such as oleic acid. Polyunsaturated fatty acids were also labelled but these were not

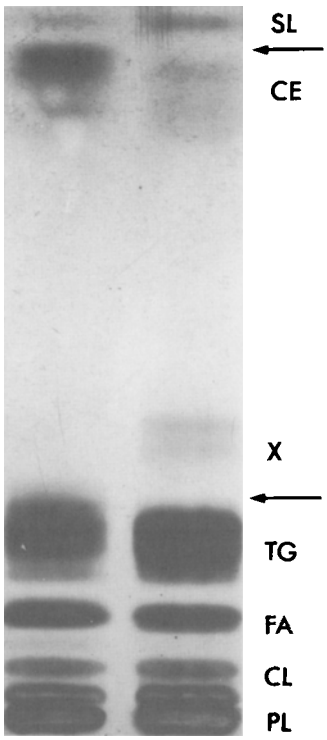

FIG. 1. Incorporation of [2-^{14}C]acetate into fibroblast neutral lipids. Fibroblast cultures were pulsed with [2-^{14}C]acetate as described in the text and the lipids extracted. The neutral (nonpolar) lipids were separated by thin-layer chromatography as described in the Materials and Methods section and the labelled products visualized by autoradiography. The amount of radioactivity applied to the different lanes was not the same. From left to right are a Zellweger then a control cell extract. X refers to an unidentified lipid; SL, to squalene; CE, to cholesteryl esters; TG, to triacylglycerol; FA, to unesterified fatty acids; CL, to cholesterol; and PL, to phospholipids. The arrows mark the position of the more hydrophobic molecular species of triacylglycerols and cholesteryl esters, which are evident in the Zellweger, but not the control, extract.

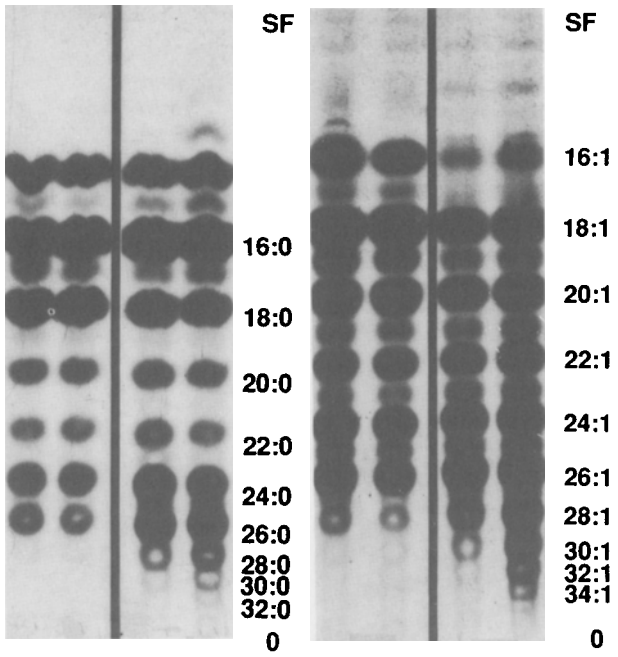

FIG. 2. Reverse phase thin-layer chromatography (TLC) of triacylglycerol-derived fatty acids. Fibroblasts were pulsed with labelled acetate as described in the text. Labelled saturated and monounsaturated fatty acids were isolated from triacylglycerols and chromatographed on reverse phase plates as described in the text. The amount of radioactivity applied to the different lanes was not the same. The figures show the autoradiographs produced after TLC. The figure on the left shows the labelled saturated fatty acids and the one on the right the labelled monounsaturated fatty acids. From the left to right of each figure are the triacylglycerol-derived fatty acids from two control then two Zellweger cell lines. The identity of the fatty acids is shown on the right of each figure, the origin on the TLC plate is indicated by an O and the solvent front by SF.

further characterized. The labelled fatty acids recovered also included a number of longer chain saturated and monounsaturated fatty acids with carbon chain lengths up to 32 (Fig. 2). The fatty acids recovered from Zellweger cells differed significantly from control cells in that they contained increased proportions of saturated and monounsaturated VLCFA with carbon chain lengths of at least up to 34 and a reduced proportion of label in stearic and oleic acids (Table 2, Fig. 2). Similar labelling patterns were observed after a 7-d chase (data not shown).

Labelled fatty acids incorporated into cholesteryl esters in control cells after a three-day incubation with labelled acetate also included a variety of products with palmitic acid predominating (Fig. 3, Table 3). Smaller amounts of saturated VLCFA were also present. Monounsaturated fatty acids were also detected, the main product being oleic acid. The labelling pattern for Zellweger cells differed primarily in that there was a greater proportion of label in monounsaturated VLCFA.

DISCUSSION

Our data demonstrate that human skin fibroblasts in culture actively incorporate the label from [2-^{14}C]acetate into a variety of neutral lipids and phospholipids with most of the label appearing in esterified fatty acids. Most of the label was incorporated into triacylglycerols with significant amounts in a variety of phospholipids and only minor amounts in free and esterified cholesterol. Our analysis of triacylglycerol and cholesteryl ester fatty acids was prompted by chromatographic evidence of the presence of more hydrophobic molecular species (and possibly VLCFA-containing molecular species of both of these lipid classes), in lipid extracts derived from Zellweger's syndrome cells grown in the presence of the label. The accumulation of labelled saturated and monounsaturated VLCFA in these two fractions in Zellweger cells almost certainly reflects the impairment in peroxisomal function reported in these cells (16). Cells from patients with X-linked ALD, a peroxisomal disorder characterized by a block in peroxisomal β-oxidation, have been reported to have increased 18:0 elongase activity (17,18), indicating that the increased microsomal elongation of fatty acids

TABLE 2

Incorporation of Label from [2-^{14}C]Acetate into Triacylglycerols[a]

	Experiment 1		Experiment 2		Experiment 3	
Fatty acids	Control (%)	Zellweger (%)	Control (%)	Zellweger (%)	Control (%)	Zellweger (%)
14:0	7.0	5.9	6.1	5.5	2.2	1.6
16:0	65.9	56.7	63.2	62.1	59.7	55.5
18:0	12.8	7.6	14.7	6.3	23.1	6.5
18:1	3.6	2.5	6.1	2.0	2.3	2.6
20:0	1.1	1.1	0.8	0.8	1.4	1.1
20:1	1.2	2.5	1.6	2.3	0.9	1.7
22:0	0.5	0.7	0.4	0.5	0.9	1.0
22:1	0.4	1.3	0.5	1.1	0.4	—
24:0	1.6	3.7	1.2	2.2	2.0	2.3
24:1	0.9	3.6	0.9	2.5	0.8	5.1
25:0	0.2	0.5	—	0.3	0.2	—
25:1	0.1	0.7	0.2	0.6	—	—
26:0	0.9	3.5	0.5	3.7	1.1	4.1
26:1	0.8	5.0	0.5	4.5	0.8	12.7
28:0	—	0.4	—	0.6	0.1	0.7
28:1	—	0.5	—	0.9	0.2	3.1
30:0	—	—	—	0.2	0.1	0.4
30:1	—	—	—	0.4	0.1	0.2
32:0	—	—	—	—	—	0.2
32:1	—	—	—	0.1	0.1	—
Other	3.0	3.8	3.3	3.5	3.7	1.1

[a]Fibroblast cell cultures were pulsed with [2-^{14}C]acetate as described in the text. The data are from three separate experiments (with different Zellweger and control lines for each experiment) and represent the percent distribution of recovered label in individual saturated and monounsaturated fatty acids isolated from triacylglycerol. Small amounts of label were recovered in odd carbon chain length fatty acids, and these are referred to as "Other fatty acids." Amounts of less than 0.1% are indicated by a dash.

TABLE 3

Incorporation of [2-^{14}C]Acetate into Cholesteryl Esters[a]

	Experiment 1		Experiment 2	
Fatty acids	Control (%)	Zellweger (%)	Control (%)	Zellweger (%)
14:0	4.8	4.4	6.3	4.0
16:0	34.9	35.2	34.2	19.4
18:0	14.0	7.8	11.0	4.4
18:1	3.8	2.0	4.3	1.8
20:0	2.3	1.2	1.7	0.8
20:1	3.7	2.1	3.8	1.7
22:0	2.2	1.1	1.3	1.2
22:1	3.0	2.3	2.1	5.1
24:0	4.5	4.2	6.4	5.9
24:1	5.7	6.3	5.7	12.9
25:0	—	—	1.5	—
26:0	3.8	5.7	6.2	8.8
26:1	3.7	10.9	5.8	16.9
28:0	0.2	1.7	0.7	1.2
28:1	0.4	3.4	0.7	2.5
30:0	0.2	0.4	—	0.2
30:1	0.2	1.4	0.7	0.4
32:0	—	1.0	—	—
32:1	0.1	1.0	—	—
34:0	0.1	0.9	—	—
Other	12.4	7.0	7.6	12.8

[a]Fibroblast cultures were pulsed with [2-^{14}C]acetate as described in the text. The data are from two separate experiments (with different Zellweger and control lines for each experiment) and represent the percent distribution of recovered label in individual saturated and monounsaturated fatty acids isolated from cholesteryl esters. Small amounts of label were recovered in odd carbon chain length fatty acids and these are referred to as "other fatty acids." Amounts of less than 0.1% are indicated by a dash.

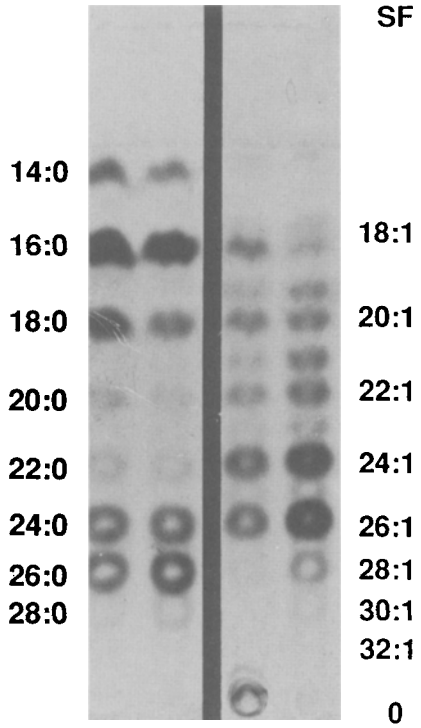

FIG. 3. Reverse phase thin-layer chromatography (TLC) of cholesteryl-ester derived fatty acids. Fibroblasts were pulsed with labelled acetate, and the saturated and monounsaturated fatty acids were isolated from the cholesteryl esters and subjected to reverse phase TLC as described in the text. The amount of radioactivity applied to the different lanes was not the same. The labelled fatty acids were visualized by autoradiography. The left panel shows the saturated fatty acids and the panel on the right the monounsaturated fatty acids. From the left to right of each panel are a control and then a Zellweger sample. The identity of the fatty acids is indicated as well as the origin (O) and solvent front (SF) on the TLC plate.

was in some way related to the peroxisomal defect. More recent studies have shown that there is an increased elongation of exogenous saturated VLCFA in Zellweger's syndrome and X-linked adrenoleukodystrophy fibroblasts (7,8,19). It has been postulated that the increased elongation of exogenous VLCFA may result as a consequence of its reduced breakdown (and therefore increased substrate availability). Our data demonstrate that there is also a greatly increased incorporation of labelled acetate into saturated and monounsaturated VLCFA. However, the identity of the endogenous fatty acids which are elongated to produce the labelled VLCFA and the elongases which catalyze these reactions are unknown.

Although saturated and monounsaturated VLCFA with up to 30 carbon atoms are important components of normal brain (20), VLCFA with more than 24 carbon atoms are only trace components of human skin fibroblasts with 26:0 comprising less than 0.1% while 28 and longer carbon chain length fatty acids are not detectable (21). We were surprised therefore to find some label associated with fatty acids with more than 26 and up to 32 carbon atoms even in normal fibroblasts (Fig. 2). The most likely explanation for the inability to detect endogenous levels of these fatty acids by the usual gas chromatographic procedures is that these longer chain fatty acids must turn over sufficiently rapidly to prevent any accumulation. They are probably synthesized *via* the sequential addition of 2-carbon units derived from acetate but their significance remains uncertain. They may simply represent secondary products of carbon chain elongation of shorter chain fatty acids although it is also possible that they may have some specific cellular function. Whether their function is the same or similar to that of the corresponding n-3 and n-6 polyenoic VLCFA found in brain (22), retina (23) and spermatozoa (24) remains to be determined.

The findings reported here provide further evidence for the importance of peroxisomal function in lipid metabolism. While it is clear that fatty acid oxidation represents a major function, it is perhaps not as well appreciated that the organelle plays a direct role in the synthesis of certain lipid classes, *e.g.*, ether lipids, as well as providing an important source of acetate for fatty acid synthesis occurring in other parts of the cell. The effect of the loss of this source of acetate, as occurs in peroxisomal-deficient cells, is not known, but it seems likely that the loss may be compensated for in other parts of the cell. It is possible that the disturbances in acetate metabolism we have observed are a reflection of these compensatory changes.

ACKNOWLEDGMENT

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Docosahexaenoic Acid Increases Permeability of Lipid Vesicles and Tumor Cells

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Docosahexaenoic acid (DHA), a long-chain polyunsaturated ω3 fatty acid, is tested to determine its mode of action as an anti-cancer agent. We demonstrate that DHA can increase the permeability of phospholipid vesicles, as monitored by vesicle swelling in isomolar erythritol and leakage of sequestered carboxyfluorescein, and T27A tumor cells, as monitored by swelling in isomolar erythritol and release of sequestered ^{51}Cr. DHA was incorporated into lipid vesicles as either the free fatty acid or as 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine. DHA was incorporated into the tumor cells by fusion with vesicles made from the mixed-chain phosphatidylcholines. DHA is demonstrated here to be much more effective in increasing permeability than is oleic acid, the major unsaturated fatty acid normally found in tumor plasma membranes. It is proposed that incorporation of DHA makes tumor plasma membranes substantially more permeable, which may explain, in part, its anti-tumor properties.
Lipids 28, 103-108 (1993).

Long-chain ω3 polyunsaturated fatty acids are believed to play a role in the prevention of a number of serious human afflictions (1) including cancer (2,3), heart disease (4) and arthritis (5). Their deficiency may also be involved in learning disabilities associated with early brain development (6,7) and may even play a role in multiple sclerosis (8). Involvement in such a broad spectrum of abnormal and pathological states implies a basic underlying molecular role for these fatty acids. Two fundamentally different nonexclusive roles have been proposed: ω3 fatty acids may affect membrane structure or they may serve as precursors to potent bioactive substances (*e.g.*, eicosanoid hormones). We have been investigating how one ω3 fatty acid, docosahexaenoic acid (DHA), may alter the physical structure of phospholipid bilayer membranes, producing changes in membrane environment and altering cell response.

Since the number and location of unsaturations are known to determine the conformation of the fatty acid chain, it would not be surprising if incorporation of a polyunsaturated fatty acid, such as DHA (22:6ω3), into membrane phospholipids might severely affect lipid packing and produce major changes in membrane function (9). However, by either monitoring the phase transition temperatures of mixed chain phosphatidylcholine (PC) molecules (10) or by using a variety of fluorescent membrane probes (11), no significant differences in membrane properties between α-linolenic acid (18:3ω3) and DHA could be detected. From these and other reports, it has become evident that while DHA significantly decreases the phase transition temperature and increases membrane fluidity of PC relative to saturated phospholipids, these properties are similar to membranes containing other, less unsaturated fatty acids. To understand why organisms would expend the energy required to produce a fatty acid that is so susceptible to oxidation, the effect of DHA on other membrane properties must be investigated. Here we report the effect of 18:0/22:6 PC on ion permeability of phospholipid vesicles and a living tumor cell (T27A).

EXPERIMENTAL PROCEDURES

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0/16:0 PC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18:0/18:0 PC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18:0/18:1 PC) and egg PC were purchased from Avanti Polar Lipids (Alabaster, AL). Oleic acid and DHA were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (18:0/22:6 PC) was synthesized as described by Ehringer *et al.* (11). DHA anhydride was purchased from Nu-Chek-Prep (Elysian, MN), while carboxyfluorescein was from Molecular Probes (Eugene, OR).

Sample preparation. Lipids used in the preparation of vesicles were dissolved in chloroform and dried under a stream of nitrogen followed by vacuum pumping for a period of 10-12 h. Multilamellar vesicles (MLV) were made by hydrating the lipids in appropriate aqueous buffer and by vortexing the aqueous dispersion above the phase transition temperatures of the lipids for a period of 10 min. Small unilamellar vesicles (SUV) were prepared by sonication of the MLV on ice using a Heat Systems W-380 Cell Disruptor (level 5; Farmingdale, NY). All lipids were stored in the dark under an atmosphere of N_2 at −20°C prior to use. Lipids were occasionally analyzed before and after sonication by thin-layer chromatography (12) and gas chromatography (GC) (13) to determine if degradation of the lipids had occurred.

Erythritol permeability of MLV. The permeability of MLV to erythritol was followed spectrophotometrically with a Perkin-Elmer Lambda 4C ultraviolet (UV)/Visible Spectrophotometer interfaced with a Perkin-Elmer (Norwalk, CT) 7700 Professional Computer run by a PECUV program. MLV were hydrated in 40 mM glucose and 20 mM Na_2HPO_4 (pH 7.0) above the phase transition temperature of the lipid and equilibrated to the swelling temperature (25°C for the mixed chain PC or 47°C for the vesicles composed of 18:0/18:0 PC and free fatty acids). Approximately 200 μL of the lipid solution (10 mM) was rapidly injected into the swelling buffer (40 mM erythritol, 20 mM Na_2HPO_4, pH 7.0), and the swelling of the vesicles due to erythritol influx was monitored by measuring the absorbance at 350 nm. The initial swelling velocity

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Abbreviations: CF, carboxyfluorescein; DHA, docosahexaenoic acid; GC, gas chromatography; MLV, multilamellar vesicles; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SUV, small unilamellar vesicles; UV, ultraviolet; 16:0/16:0 PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 18:0/18:0 PC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; 18:0/18:1 PC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:0/22:6 PC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

of the MLV (d(1/A)/dt%), measured for the first 20–30 s, is known to be directly proportional to erythritol permeability (14). Most permeability measurements were conducted in the liquid crystalline state of the lipids.

Carboxyfluorescein leakage from SUV. Measurements of ionic permeability of 18:0/18:1 PC and 18:0/22:6 PC SUV were followed by monitoring the increase in fluorescence intensity from carboxyfluorescein (CF) leakage (15). MLV were made by hydrating the lipids in 60 mM CF, 30 mM KCl, 10 mM Na_2HPO_4 (pH 7.0) above the phase transition temperature. SUV were made as described above. External nonsequestered CF was removed by passing the suspension down a column (1.5 cm × 30 cm) of Sephadex G-50 equilibrated with 90 mM KCl and 10 mM Na_2HPO_4 (pH 7.0). Aliquots of the vesicles (100 μL) were immediately mixed with 2.5 mL of 90 mM KCl/10 mM Na_2HPO_4, pH 7.0. Vesicles were kept on ice until the experiment was initiated to keep CF sequestered. The efflux of self-quenched CF from the SUV was monitored by an increase in fluorescence with excitation at 490 nm and emission at 520 nm using a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer interfaced with a Perkin-Elmer 7700 Professional Computer. Temperature in the cuvettes was kept at 25°C with a circulating water bath. Maximum fluorescence was determined by addition of 100 μL of 5% Triton X-100, and subsequent CF leakage was calculated as follows:

$$\% \text{ CF leakage} = F - F_i/F_{max} (100) \quad [1]$$

where F is the fluorescence intensity at time t, F_i is the initial fluorescence at time zero, and F_{max} is the maximum fluorescence.

T27A cells. T27A is a non-T, non-B leukemia cell isolated from a BALB/c mouse infected with N-tropic murine leukemia virus (16). This cell line can be maintained both as an ascites tumor and in culture. To passage *in vivo*, each mouse was inoculated with 10^5 T27A cells i.p. 7 d before the ascites were harvested by peritoneal lavage. Ascitic tumor cells were freed of erythrocytes by lysis in 0.155 M NH_4Cl, 10 mM $KHCO_3$, 1 mM disodium ethylenediaminetetraacetic acid (pH 7.4) for 5 min at room temperature and were washed in Hanks' balanced salt solution. Cell lines were also maintained in culture in supplemented RPMI 1640 medium (plus antibiotics, 25 mM *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] buffer and 2 mM glutamine) with 20% bovine calf serum (HyClone Laboratories, Logan, UT); under these conditions, the T27A line has a generation time of 12 to 15 h and grows to a maximum density of 1–2 × 10^6 cell/mL.

Vesicle fusion with T27A cells. SUV were made in phosphate buffered saline (PBS), pH 7.4, and fused with 1 × 10^7 T27A cells by incubation at room temperature for 10 min, 1 h or 2 h. Unfused vesicles were removed by one wash in cold 10% bovine serum albumin in PBS and three washes in PBS + 5% fetal bovine serum. Cell numbers were determined with a hemacytometer, and viability was checked by trypan blue exclusion.

Swelling of T27A cells. Cells fused with the vesicles were immediately subjected to isotonic swelling in erythritol. Briefly 3 × 10^6 cells of the fused T27A cells incubated at the swelling temperature (37°C) were injected into 3.0 mL of 175 mM sucrose/75 mM erythritol/20 mM Na_2HPO_4 (pH 7.4) at 37°C. The influx of erythritol and subsequent swelling of the cells was monitored at 420 nm with a Perkin-Elmer Lambda 4C UV/Visible Spectrophotometer. The results presented are the average of 20 experiments.

GC. Cells and vesicles to be analyzed by GC were placed in a separatory funnel, and the lipids were extracted three times with 80 mL of $CHCl_3/CH_3OH$ (2:1, vol/vol) along with 10 mL of a 0.5 N NaCl solution. The lipid extracts were combined and rotovaporated to a small volume. Saponification of the lipid material was accomplished by addition of 0.5 N NaOH in methanol and refluxing the mixture for 1 h. Fatty acids were esterified by addition of BF_3/methanol and refluxing again for an additional 30 min. Methyl esters were extracted three times by addition of 50 mL of hexane. The extracts were combined and rotovaporated to a small volume. A 2.0 μL sample of the methyl esters was then analyzed with an SRI Gas Chromatograph equipped with a flame ionization detector interfaced to a Micro Express 386 Computer (Santa Ana, CA). The 6′ × 1/8″ column was packed with 10% SP-2330 on 100/120 Chromosorb WAW. The initial oven temperature was 190°C while final oven temperature was 225°C after 25 min. Peaks were identified by direct comparison with the retention times of methyl ester standards (Nu-Chek-Prep, Elysian, MN).

^{51}Cr release. T27A cells (5 × 10^6) were incubated with 150 μCi of sodium [^{51}Cr]chromate (>500 mCi/mg; New England Nuclear, Boston, MA) in RPMI plus 5–10% serum and then washed three times in PBS + 5% serum. The cells were diluted to 5 × 10^4 cells/mL with RPMI + 5% serum, and 100 μL of the cell suspension was incubated in triplicate with 100 μL of medium (for spontaneous release) or detergent (for maximum release) for 4 h at 37°C in a humidified 7.5% CO_2 atmosphere.

RESULTS

Previous reports have indicated that DHA esterified to the *sn*-2 position of PC can increase bilayer permeability of nonelectrolytes (11,17). In Figure 1 we confirm these reports for the mixed chain 18:0/22:6 PC. The PC containing DHA is about 2.4 times more permeable to erythritol than is the corresponding PC containing oleic acid (18:0/18:1 PC). The disaturated PC (18:0/18:0 PC) which exists in the gel state at 25°C is almost impermeable. When DHA as the free fatty acid was incorporated at 20 membrane mol% into 16:0/16:0 PC bilayers, permeability to erythritol was dramatically increased (Fig. 2). Again oleic acid enhanced permeability but to a much lesser extent than did DHA (DHA/oleic acid permeability ratio = 3.0).

The effect of DHA on permeability to the anionic CF is shown in Figure 3. The lipid vesicles are composed of either egg PC (control) or of 80 mol% egg PC to which is added 20 mol% 18:0/18:1 PC or 18:0/22:6 PC. CF leakage is greater for the DHA-containing PC than it is for the oleic acid-containing PC. After 30 min the ratio of DHA/oleic acid permeability is about 2.0.

To assess the effect of DHA on the permeability of living T27A tumor cells, the tumor cells were modified by fusion with lipid vesicles composed of 18:0/22:6 PC and, for comparison, vesicles composed of 18:0/18:1 PC and the nonfusogenic control 16:0/16:0 PC. As shown in Figure 4 (insert), incorporation of DHA increases as a function of fusion time for 2 h at which time it comprises about 5% of the total membrane fatty acids. The cultured T27A cells

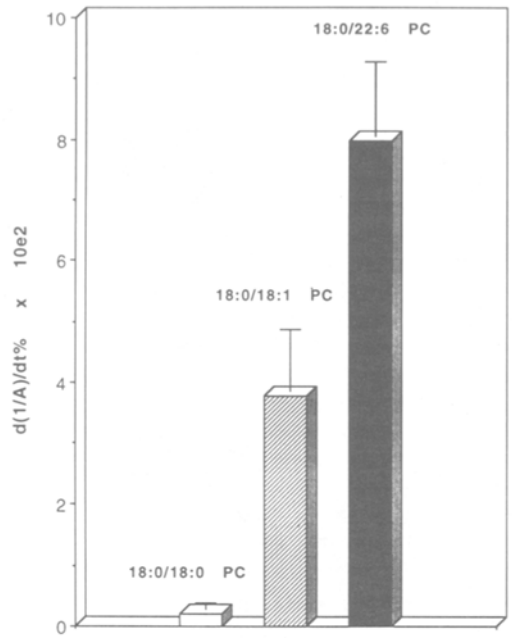

FIG. 1. Erythritol permeability for multilamellar vesicles (MLV) composed of 18:0/18:0 phosphatidylcholine (PC), 18:0/18:1 PC and 18:0/22:6 PC (25°C). MLV stock solutions (10 mM) were made in 40 mM glucose, 20 mM Na_2HPO_4, pH 7.0 and rapidly injected into 2.0 mL of the swelling buffer (40 mM erythritol, 20 mM Na_2HPO_4, pH 7.0). Lipid concentration was 1 mM, and vesicle swelling was followed at 25°C by absorbance at 350 nm. At this temperature 18:0/18:0 PC is in the gel state where its permeability is extremely low. Results are the mean of 20 experiments and are significant to the 0.001 level as determined by analysis of variance.

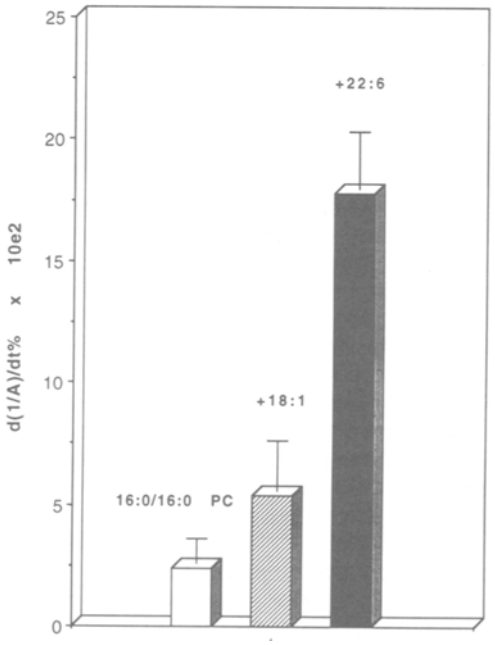

FIG. 2. Effect of oleic acid and docosahexaenoic acid as free acids on erythritol permeability of 18:0/18:0 phosphatidylcholine (PC) multilamellar vesicles (47°C). Experimental procedure is as outlined for Figure 1. At 47°C 18:0/18:0 PC is in the liquid crystal state. The fatty acids are incorporated at 20 membrane mol%. Results are the mean of 20 experiments and are significant to the 0.001 level as determined by analysis of variance.

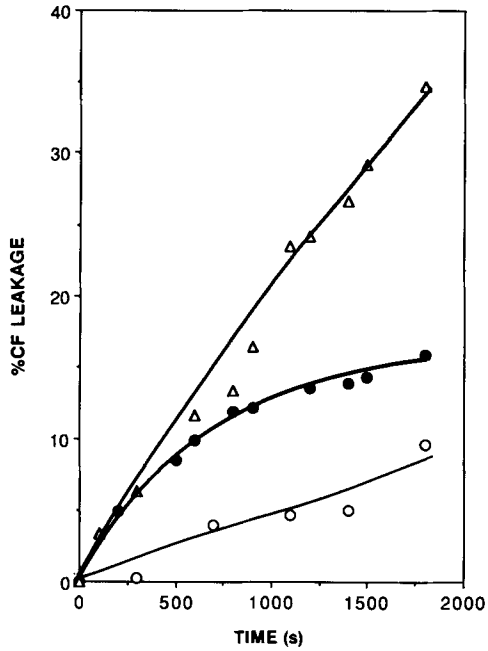

FIG. 3. Release of sequestered carboxyfluorescein (CF) from egg phosphatidylcholine (PC) small unilamellar vesicles (SUV) (30°C). Stock solutions of SUV composed of 100 mol% egg PC (open circles); 80 mol% egg PC, 20 mol% 18:0, 18:1 PC (closed circles); and 80 mol% egg PC, 20 mol% 18:0, 22:6 PC (open triangles) were made in 60 mM CF, 30 mM KCl, 10 mM Na_2HPO_4, pH 7.0. After nonsequestered CF was removed on a Sephadex G-50 column, aliquots of CF-loaded SUV were rapidly injected into 2.5 mL of 90 mM KCl/10 mM Na_2HPO_4, pH 7.0. Lipid concentration was between 200 to 400 μM, and the fluorescent intensity monitored at 520 nm (excitation 490 nm). Results are the average of three determinations and are significant to the 0.001 level by analysis of variance.

had essentially no DHA before fusion. As DHA accumulated in the tumor membranes, the cells became more permeable to erythritol (Fig. 4). When DHA represented 5% of the total fatty acids, T27A permeability was about triple that of the unaltered cells. By the first reported time period (10 min after fusion was initiated), it was clear that 18:0/22:6 PC fused much better with the T27A cells than did 18:0/18:1 PC (Fig. 5, insert). But even when incorporated at equivalent levels, DHA enhanced permeability to a much greater extent than did oleic acid (Fig. 5). For the permeability results presented in Figure 5, the T27A cells were fused with 18:0/18:1 PC vesicles at 2.0 mg/mL while incubation with the 18:0/22:6 PC vesicles was at 0.5 mg/mL. The insert of Figure 5 shows that fusion of 0.5 mg/mL of 18:0/22:6 PC produces an equivalent incorporation of lipid into T27A cells as does 2.0 mg/mL of 18:0/18:1 PC. The effect of 18:0/18:1 PC and 18:0/22:6 PC on membrane permeability was confirmed by monitoring the leakage of sequestered ^{51}Cr from T27A cells (Fig. 6). The DHA-containing cells were about 1.9 times more permeable to ^{51}Cr than the 16:0/16:0 PC (unaltered) controls and 1.5 times more permeable than the oleic acid-altered cells.

DISCUSSION

The fatty acids of phospholipids comprise the hydrophobic core of membranes and are known to affect a wide

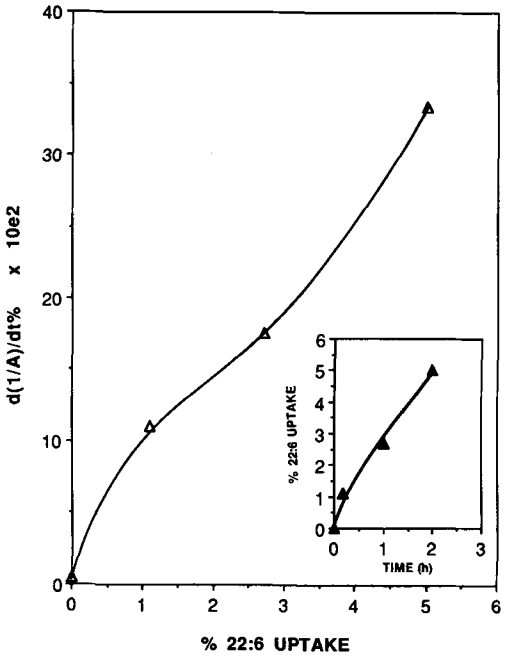

FIG. 4. Permeability of T27A cells fused with 0.5 mg/mL 18:0/22:6 phosphatidylcholine (PC) to erythritol, expressed as the rate of cell swelling (d(1/A)/dt%) as a function of docosahexaenoic acid (DHA) incorporation. 3×10^6 cells were injected into the swelling buffer (175 mM sucrose, 75 mM erythritol, 20 mM Na_2HPO_4, pH 7.4, 37°C). Erythritol-induced swelling was followed by absorbance at 420 nm. Insert: Uptake of DHA into T27A cells as a function of fusion time with 18:0/22:6 PC (0.5 mg/mL). Sample correlation coefficient for the swelling velocity *vs.* % 22:6 uptake is r = 0.988, while the insert is r = 0.991.

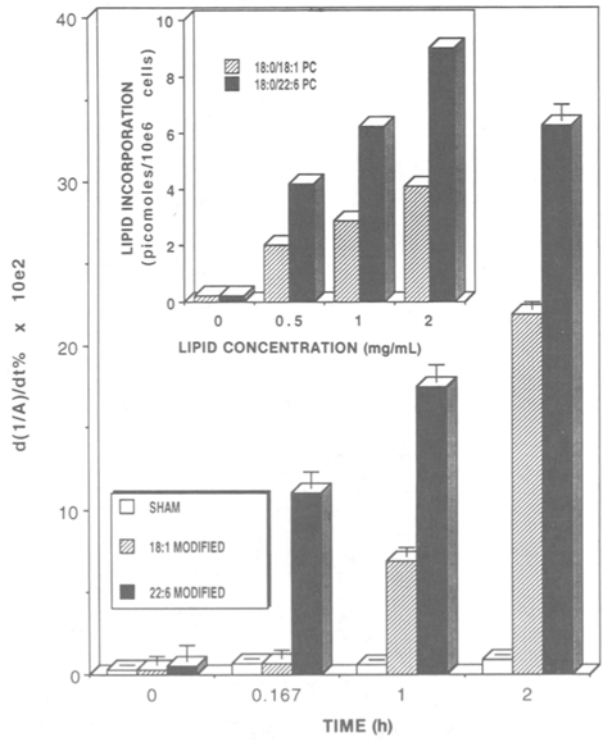

FIG. 5. Permeability of erythritol of T27A cells fused with 2.0 mg/mL 16:0/16:0 phosphatidylcholine (PC) (SHAM), 2.0 mg/mL 18:0/18:1 PC (18:1 MODIFIED) or 0.5 mg/mL 18:0/22:6 PC, 2.0 mg (22:6 MODIFIED) expressed as the rate of cell swelling (d(1/A)/dt%) *vs.* fusion time. Insert: Lipid incorporation as 18:0/18:1 PC vesicles or 18:0/22:6 PC vesicles are fused with T27A tumor cells. Permeability measurements are the average of 10 runs with $P < 0.001$ at all times tested.

variety of membrane properties. The extent of fatty acid unsaturation in particular has been shown to alter membrane order and fluidity, thickness, domain size and stability, permeability, lipid phase and, through interaction with membrane proteins, even biochemical activity (9,18–22). Of particular interest to us is the long-chain ω3 fatty acid DHA (DHA, 22:6), which is one of the most unsaturated lipids in biological systems and is mainly found at specialized locations where it probably provides specific, but as yet undefined, functions (9). Lower levels of DHA are found in most other membranes where its concentration may be further enhanced through diet (23–25). It appears that in membranes containing extremely high levels of DHA naturally, such as rod outer segment (26), sperm (27) and the gray matter of brain (28), DHA is found primarily in phosphatidylserine and phosphatidylethanolamine (PE) (1). In contrast, dietary supplementation produces increased levels of DHA in other membranes and mainly as a component of PC and PE.

The double bond effects, however, are not additive, and these changes may not necessarily be reflected in large alterations in general membrane "fluidity." Initially it was assumed that DHA-containing phospholipids would exhibit significantly decreased phase transition temperatures compared to the less unsaturated phospholipid homologs and so would impart to membranes a substantial increase in fluidity. Surprisingly this was not found to be true. Deese *et al.* (10) reported that 16:0/22:6 PC actually had a higher T_m (−3°C) than did 16:0/16:1 PC (−10°C). Recently we observed that "fluidity" of DHA-containing PC membranes, as monitored by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene and a series of anthroloxy stearic acid probes, is increased relative to PC membranes composed of saturated acyl chains (11). However, bilayers composed of PC containing stearic acid (18:0) in the *sn*-1 position and either oleic (18:1), α-linolenic acid (18:3) or DHA (22:6) in the *sn*-2 position have very similar "fluidities." This is in agreement with several studies that showed that accumulation of DHA in biological membranes does not necessarily affect membrane fluidity (21). In contrast, Salem *et al.* (29) reported that DHA makes phosphatidylserine bilayers more "fluid" although other explanations for the results have been offered (9). The effect of DHA on membranes thus may be related to the polar head group of the phospholipid with which the fatty acid is associated. The results reported here involve membrane supplementation with DHA-containing PC and demonstrate that despite exhibiting fluidities typical of most unsaturated membranes, the DHA-enriched membranes have greatly enhanced permeabilities.

Here we have chosen 18:0/18:1 PC as a fusable control lipid to compare with 18:0/22:6 PC. Our analysis of T27A tumor cell plasma membranes (Ehringer, W., and Stillwell, W., unpublished results) show 11.7% oleic acid and only 0.4% linolenic acid. We reason, therefore, that addition of 18:0/18:1 PC will not severely alter the normal

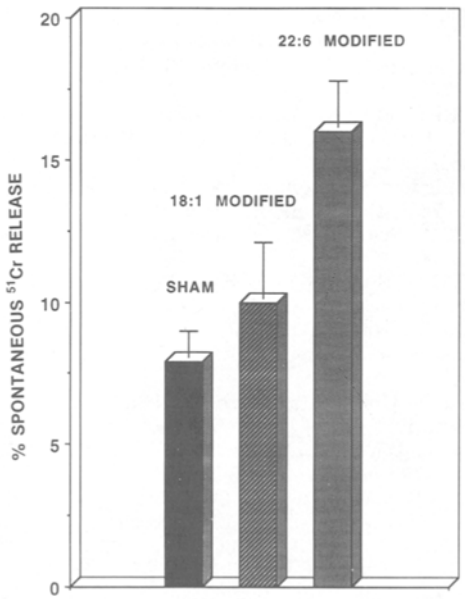

FIG. 6. Release of sequestered ^{51}Cr from lipid modified T27A cells. The cells (5×10^6) were incubated with 150 μCi of sodium (^{51}Cr) chromate and fused with either 18:0/22:6 phosphatidylcholine (PC) (22:6 MODIFIED), 18:0/18:1 PC (18:1 MODIFIED) or no lipid (SHAM). The percentage of spontaneous release was determined by addition of detergent. Results are the mean values of three experiments. Statistically significant differences were tested by analysis of variance for A) 22:6/18:1 modified cells and B) 18:1/sham: A) P, 0.0001; B) $P = 0.1119$.

composition of T27A plasma membranes but will indicate if the process of membrane fusion causes membrane damage resulting in enhanced permeability. Although the small amount of tumor cells used in the experiments presented here precluded us from performing a direct lipid analysis on the T27A plasma membranes, we nevertheless are convinced that the fused 18:0/22:6 PC resides at least in part with the cell surface (plasma) membrane. We have shown that treatment of murine T-cell leukemia cells with 18:0/22:6 PC lipid vesicles results in a dose-dependent change in epitope expression on a plasma membrane protein (30). The most reasonable explanation of these data is that 18:0/22:6 PC fused with the plasma membrane, altered membrane structure and affected the expression of membrane proteins. The monoclonal antibodies used to detect epitope expression monitor events only at the plasma membrane. Additionally, the increased expression of one epitope concurrently with decreased expression of another epitope on the same protein argues against an effect of 18:0/22:6 PC on protein synthesis.

Acyl chain composition of phospholipids has for years been known to greatly affect membrane permeability. In general, a direct relationship was found between the level of unsaturation and the magnitude of membrane permeability (17,31). If the studies on fatty acids with 1, 2 or 3 double bonds can be extrapolated to 6 double bonds, one would predict that DHA might produce extremely permeable membranes. In fact the rod outer segment membrane, which is known to be highly enriched in DHA (26), is also very permeable to Na^+ (32). However, only a few reports on the direct effect of DHA on membrane permeability have actually appeared. Chow and Jondal (33) showed that at high levels (50 μM or more in the bathing solution) free DHA increased cell permeability to ^{51}Cr. Since under normal physiological conditions free fatty acids are found only in trace amounts in membranes, this report does not address the role of DHA as it would normally be found in biological membranes. Burns and Spector (34-36) reported that dietary DHA make L1210 murine ascites lymphoblastic leukemia cells more permeable to the anti-cancer drugs mitoxantrone and doxorubicin. These drugs are thought to cross cell membranes by diffusion through the lipid phase (34). The biochemical species containing DHA was not reported for these experiments. In the experiments reported here, we use two very different techniques, cell swelling in isomolar erythritol and spontaneous release of sequestered ^{51}Cr to directly confirm that DHA esterified to the *sn*-2 position of PC enhances permeability of the tumor cell T27A.

DHA has also been shown to increase permeability of well-defined phospholipid vesicles. Demel *et al.* (17) reported that PC lipid vesicle permeability to glucose, erythritol and glycerol increased in proportion to the area/molecule of the membrane phospholipids. DHA in mixed chain PC produced a very permeable bilayer membrane in their system. Ehringer *et al.* (11) confirmed these finding showing that 18:0/22:6 PC-containing vesicles were far more permeable to erythritol than were 18:0/18:3 PC vesicles. The experiments reported here agree with the previous reports that DHA incorporated into PC bilayers increases permeability.

For several reasons we have chosen to add DHA as a mixed chain PC to tumor cells. Dietary DHA is known to readily accumulate in the *sn*-2 position of several phospholipids and mostly PC (23). The *sn*-1 chain of most PC is either palmitic acid (16:0) or stearic acid (18:0) (10). Therefore by fusing the tumor cell plasma membranes with 18:0/22:6 PC, we are enriching the cells with DHA acylated to PC, as it would be found in most biological membranes. Although there is a large body of evidence that DHA can have significant anti-tumor properties, there is little known about the possible mechanism of action. The experiments presented here indicate that the permeability of T27A cells can be substantially enhanced by accumulation of as little as 5% DHA into their membranes. It is also known that within a few weeks mice on dietary fish oils can increase the membrane levels of DHA to levels comparable to our permeability studies (34). Dietary experiments however can have serious interpretational drawbacks as the ratios of the various phospholipid types and other membrane components such as sterols may change as a function of the diet. Fusion experiments like those reported here alter the concentration of only a single membrane species and so data interpretation is more direct.

The increase in membrane permeability affected by DHA-containing PC reported here may be related to the well-documented anti-cancer properties of dietary fish oils. Kitada *et al.* (37) have reported that lipid mobilization in tumors is directed toward membrane synthesis and not energy production. We would therefore expect that DHA may accumulate faster in tumors than in the surrounding host tissues. The ensuing membrane perturbations caused by DHA could render the tumor more susceptible to anticancer drugs or to destruction by the immune system.

ACKNOWLEDGMENTS

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Hypertension in Rats Does Not Potentiate Hypercholesterolemia and Aortic Cholesterol Deposition Induced by a Hypercholesterolemic Diet

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The effect of a hypercholesterolemic diet (HCD) on hyperlipemia and atherogenesis was investigated using normotensive Wistar/Kyoto rats (WKY), spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP), with systolic blood pressures increasing in that order. Feeding an HCD diet containing cholesterol, cholate and suet induced hypercholesterolemia in all the strains examined as compared with a normal diet. The plasma cholesterol levels were significantly higher in WKY than in SHR and SHRSP fed the HCD diet. The HCD diet also induced hepatic fat deposition, particularly deposition of cholesteryl esters, a slight increase in aortic cholesterol deposition, and elevation of both monoenoic/saturated fatty acid ratios and linoleate/arachidonate ratios in tissue lipids. The changes induced in the three strains by the HCD diet were not positively correlated with blood pressures. The HCD diet affected hepatic acyl-CoA:cholesterol acyltransferase and plasma lecithin:cholesterol acyltransferase activities differently in WKY and SHR which, in addition to the induction of Δ9 desaturase, may partly account for the difference in the diet-induced changes in the fatty acid compositions of plasma cholesteryl esters. The results indicate that hypertension *per se* does not stimulate the development of hypercholesterolemia and arterial cholesterol deposition induced by an HCD diet, suggesting that other factors are involved.
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Hypertension and hypercholesterolemia are generally recognized as risk factors for atherosclerosis. Accumulation of cholesteryl esters, particularly cholesteryl oleate, is a characteristic feature of atherosclerotic lesions (1–5). Monocyte-derived macrophages appear to play a key role in the atherogenic process (6), and most of the cellular cholesterol is assumed to be derived from plasma lipoproteins. In rabbits, hyperlipemia and atherosclerosis are easily induced by atherogenic diets (7–12), but normal rats rarely develop atherosclerosis or arterial fat deposition even after long-term feeding of atherogenic diets.

In contrast, spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHRSP) have been reported to rapidly develop hyperlipemia and ring-like arterial fat deposition when placed on an atherogenic diet (13–16). Therefore, it is widely accepted that hypertension is a risk factor which stimulates cholesterol infiltration into the arterial wall. However, our previous studies showed that plasma cholesterol levels were higher in normotensive Wistar/Kyoto rats (WKY) than in SHR fed a high cholesterol/cholate diet, and that arterial fat depositions were not detectable in either strain of rat (17). These discrepancies may have resulted from differences in the diets used. In the present study, we fed three strains (WKY, SHR, SHRSP) derived from Wistar rats the atherogenic diet reported to induce hypercholesterolemia and aortic fat deposition (13–16), and compared plasma, hepatic and aortic lipids. The systolic blood pressure decreased in the order SHRSP, SHR and WKY (18,19). Acyl-CoA:cholesterol acyltransferase (ACAT) and lecithin:cholesterol acyltransferase (LCAT) activities also were measured to gain some insight into the mechanism which lead to the production of different molecular species of cholesteryl esters in SHR and WKY.

MATERIALS AND METHODS

[1,2-^{3}H]Cholesterol and [9,10-^{3}H]oleic acid were purchased from New England Nuclear (Boston, MA). Radiolabeled and nonlabeled oleoyl-CoA were prepared by a modification of Seubert's procedure (20) as described elsewhere (21). Male SHR and WKY rats were obtained from Charles River of Japan (Kanagawa, Japan). SHRSP were kindly provided by Prof. Kozo Okamoto, Kinki University School of Medicine (Osaka, Japan). At 8 wk of age, these rats were fed *ad libitum* for up to 8 wk either a conventional diet (CE-2; Nippon Clea Co. Ltd., Tokyo, Japan) or a CE-2 diet supplemented with 5% cholesterol, 2% cholate, 20% suet and 1% mixed vitamins (hypercholesterolemic diet, HCD), which was prepared as described by Yamori *et al.* (14). The CE-2 diet contained (by weight): 24.9% protein, 4.5% fat, 7.5% minerals, 4.1% fiber and 50.2% non-nitrogenous compounds. The major fatty acid constituents of the CE-2 diet were linoleate (50.6%), oleate (21.9%), palmitate (14.9%) and α-linolenate (3.9%). Major fatty acids of the suet were oleate (46.5%), palmitate (23.2%), stearate (17.5%), palmitoleate (4.1%), linoleate (4.1%) and α-linolenate (0.3%).

Tail systolic blood pressure was measured at around 10:00 a.m. by the plethysmographic tail method with an apparatus produced by Natsume Co. (Tokyo, Japan). The rats were sacrificed after fasting for 17 h. Blood was sampled from the abdominal vein, and was centrifuged to separate plasma. Livers and aortas were excised. The attached fat tissues were removed from abdominal and thoracic aortas under a microscope. Samples were kept frozen at −80°C. Lipids were extracted with chloroform/methanol according to the method of Bligh and Dyer (22), and then chromatographically separated on silica gel plates (Merck 60, Merck, Darmstadt, Germany) which were prewashed with the developing solvent. Petroleum hydrocarbon (b.p. 35~60°C)/diethyl ether/acetic acid (80:30:1, by vol) was used as developing solvent for

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; Cho, cholesterol; ChoE, cholesteryl esters; FFA, free fatty acid; HCD, hypercholesterolemic diet; LCAT, lecithin: cholesterol acyltransferase; ND, normal diet; PL, phospholipid; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone spontaneously hypertensive rats; TG, triacylglycerols; WKY, Wistar/Kyoto normotensive control rats.

separating neutral lipids from phospholipids. Fatty acids of the separated fractions were analyzed as methyl esters by gas-liquid chromatography using heptadecanoic acid as internal standard. Cholesterol was quantified by gas-liquid chromatography as trimethylsilyl ether using 5α-cholestane as internal standard (23).

Rats were fasted for 16 h before preparing subcellular fractions according to the method described by Eibl *et al.* (24). Protein was determined by the method of Lowry *et al.* (25). ACAT activity was determined essentially as described by Lichtenstein and Brecher (26). The assay mixture contained 2 mM dithiothreitol, 0.1 M sodium phosphate (pH 7.4), 60 μg of microsomal protein, 16.5 μM [^{3}H]oleoyl-CoA (100,000 cpm/nmol) and 16.5 μM bovine serum albumin, all in a total volume of 0.2 mL. The assay mixture was preincubated for 5 min without microsomal protein. Incubations were started by adding enzyme and were performed with vigorous shaking at 37°C for 2 min. ACAT activity was determined by following the production of cholesteryl [^{3}H]oleate. LCAT was assayed by the method of Albers *et al.* (27). Cholesterol and cholesteryl esters were separated by silica gel thin-layer chromatography using petroleum ether/diethyl ether/acetic acid (80:30:1, by vol) as solvent. Student's *t*-test was used for statistical analysis.

RESULTS

Body weights, liver weights and blood pressures are summarized in Table 1. Feeding the HCD for 8 wk induced slight weight losses, which were the largest in the SHR strain. The test diet did not affect the blood pressure of any of the strains. Liver weights were about twofold higher in the HCD groups than in the normal diet (ND) groups. In the HCD groups, the faded liver color was typical of that of fatty liver. Aortas were stained by oil red O, but no fat depositions were noted in all the strains fed the hypercholesterolemic diet (data not shown).

The plasma lipid levels are compared in Figure 1. In the ND groups, the plasma lipid levels, and particularly the phospholipid and cholesteryl ester levels, were the highest in WKY followed by SHR, and then by the SHRSP strain.

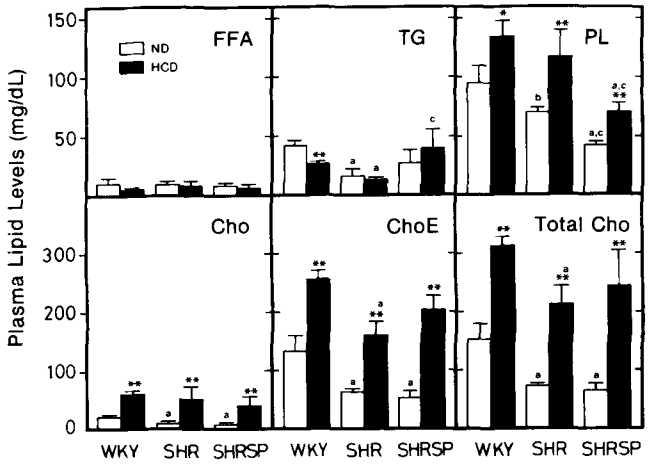

FIG. 1. Effect of a hypercholesterolemic diet on plasma lipid levels. Rats were fed the test diets for 8 wk from 8 wk of age. After fasting overnight, rats were sacrificed, and lipids were extracted from plasma with chloroform/methanol. Individual lipid classes were separated by a silica gel thin-layer chromatography and quantitated as described in the text. Cholesterol ester (ChoE), cholesterol (Cho), triacylglycerol (TG), free fatty acid (FFA) and phospholipid (PL) were determined. Normal diet group (ND, □) and hypercholesterolemic diet group (HCD, ■) in WKY, SHR and SHRSP were determined. Averages of determinations for three rats in each group (±SD) are presented. Statistical significance is shown as ** ($P < 0.01$) or * ($P < 0.05$) for the comparison of ND and hypercholesterolemic diet groups, a ($P < 0.01$) or b ($P < 0.05$) for the comparison of Wistar/Kyoto normotensive control rats (WKY) and other strains, and c ($P < 0.01$) or d ($P < 0.05$) for the comparison of spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP).

Plasma cholesterol, cholesteryl ester and phospholipid levels were elevated >3-fold, >1.9-fold and >1.4-fold, respectively, in the HCD groups as compared with the ND groups. The levels of total cholesterol in the HCD groups were 2.1-fold higher in WKY, 2.9-fold higher in SHR and 3.9-fold higher in SHRSP than in the ND groups. The difference between the ND groups and the HCD groups was the largest in SHRSP, but the total plasma cholesterol levels were the highest in WKY in both dietary groups.

TABLE 1

Effect of a Hypercholesterolemic Diet on Body Weight, Liver Weight, Blood Pressure, ACAT Activity and LCAT Activity[a]

Rat strain	Diet	Body weight (g)	Liver weight (g)	Blood pressure (mmHg)	ACAT (nmol/min/mg protein)	LCAT (nmol/h/mL)
WKY	ND	307 ± 9	10.5 ± 0.5	108 ± 2	0.16 ± 0.03	17.6 ± 0.4
	HCD	293 ± 9	19.3 ± 1.5^b	105 ± 10	0.84 ± 0.09^b	10.7 ± 1.0^b
SHR	ND	316 ± 4	12.0 ± 1.9	184 ± 6^c	0.27 ± 0.08	14.8 ± 1.0^c
	HCD	263 ± 10^{b,d}	20.5 ± 0.7^b	164 ± 16^c	0.84 ± 0.12^b	16.8 ± 2.2^c
SHRSP	ND	256 ± 10^{c,e}	10.6 ± 2.2	209 ± 12^{c,f}	n.d.	n.d.
	HCD	228 ± 11^{g,c,f}	20.1 ± 2.7^b	201 ± 17^{c,f}	n.d.	n.d.

[a]Male rats at 8 wk of age were fed a normal diet (ND) or a hypercholesterolemic diet (HCD) for 8 wk. Hepatic microsomal acyl-CoA:cholesterol acyltransferase (ACAT) activity was determined using [9,10-^{3}H]oleoyl-CoA, and plasma lecithin:cholesterol acyltransferase (LCAT) activity was assayed using [1,2-^{3}H]cholesterol. Averages of determinations (±SD) for 3 rats are presented. Statistical significance in Student's *t*-test is shown as superscript b ($P < 0.01$) or superscript g ($P < 0.05$) for the comparison of ND and HCD groups, superscript c ($P < 0.01$) or superscript d ($P < 0.05$) for the comparison of Wistar/Kyoto normotensive control rats (WKY) and other strains, and superscript e ($P < 0.01$) or superscript f ($P < 0.05$) for the comparison of spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHRSP); n.d., not determined.

Thus, the blood pressure and the total plasma cholesterol level were not positively correlated between the three strains of rats.

The amount of total cholesterol in whole plasma and whole liver were comparable in the ND groups (Fig. 2). Feeding HCD markedly increased total cholesterol in livers of all three strains; the increases were 120-fold in WKY, 150-fold in SHR and 160-fold in SHRSP. In spite of these tremendous increases in liver cholesterol, the total plasma cholesterol levels changed relatively little suggesting that secretion of hepatic cholesterol is suppressed in the HCD groups. The hepatic FFA and TG levels increased about twofold, but the PL levels were relatively unchanged in the HCD groups of the three strains (data not shown).

In the preparation of aortic tissues for lipid analysis, great care was taken to remove the surrounding fat tissues. Then, the levels of aortic phospholipid (PL), triacylglycerol (TG), free fatty acid (FFA), cholesterol and cholesteryl ester were determined. In the ND groups, the basal lipid levels were similar among the three strains. Feeding the HCD increased the aorta cholesterol levels slightly (Fig. 3); the level tended to be higher in WKY than in SHR and SHRSP. Again, the aortic cholesterol levels showed no positive correlations with the blood pressures in the three strains of rats.

Feeding the HCD induced a striking change in the fatty acid compositions of plasma and hepatic cholesteryl esters as noted previously (17). Fatty acid compositions of hepatic cholesteryl esters from WKY and SHR strains are shown in Figure 4A. The proportions of monoenoic fatty acids, especially octadecenoate, increased while those of saturated fatty acids, linoleate and arachidonate concomitantly decreased in both strains fed HCD. We previously reported that most of the octadecenoate was the Δ9 isomer, oleate (17). Similar changes induced by HCD

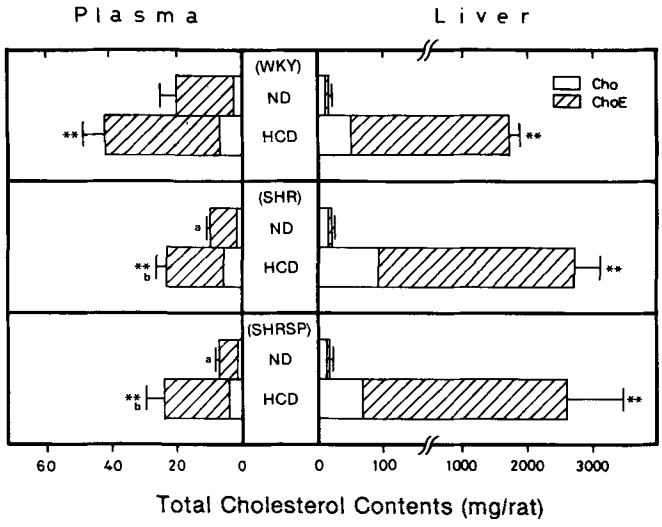

FIG. 2. Effect of a hypercholesterolemic diet on plasma and hepatic cholesterol contents. Cholesterol (Cho) and cholesteryl ester (ChoE) in plasma and liver were quantitated as described in the text. Plasma volume was assumed to be 4% of the body weight. Averages of determinations (±SD) for three rats are presented. Statistical significance is shown as ** ($P < 0.01$) or * ($P < 0.05$) for the comparison of WKY and other strains, and c ($P < 0.01$) or d ($P < 0.05$) for the comparison of SHR and SHRSP. Abbreviations as in Figure 1.

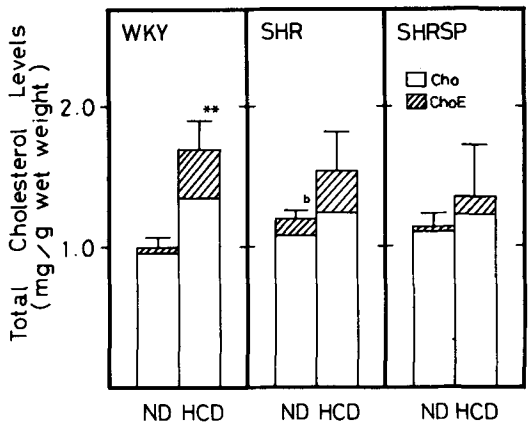

FIG. 3. Effect of a hypercholesterolemic diet on aortic cholesterol levels. Abdominal and thoracic aortas were freed of surrounding fat tissues under a microscope. Lipids were extracted and quantitated as described in the legend to Figure 1. Averages of determinations (±SD) for three rats are presented. Statistical significance is shown as ** ($P < 0.01$) for the comparison of ND and HCD groups, and b ($P < 0.05$) for the comparison of WKY and other strains. Abbreviations as in Figure 1.

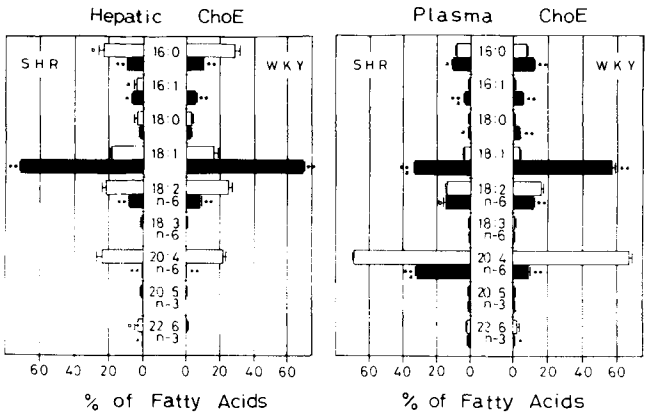

FIG. 4. Effect of a hypercholesterolemic diet on the fatty acid pattern of hepatic and plasma cholesteryl esters. Only the major fatty acids are presented. Fatty acids are designated by carbon chain length:number of double bond, and the n-6 or n-3 represents the position of the first double bond numbered from the methyl terminus. □, Normal diet group (ND); ■, hypercholesterolemic diet group (HCD). Averages of determinations (±SD) for three rats are presented. Statistical significance is shown as ** ($P < 0.01$) or * ($P < 0.05$) for the comparison of ND and HCD groups, and a ($P < 0.01$) or b ($P < 0.05$) for the comparison of WKY and SHR. Abbreviations as in Figure 1.

were also observed in other lipid classes, PL, TG and FFA. As a whole, the fatty acid patterns of hepatic cholesteryl esters were similar among the three strains of the ND groups, and HCD-induced changes also were similar among the three groups. These changes are possibly due to increased Δ9 desaturase activities (17,28) and to the impairment of elongation-desaturation activities in the formation of arachidonate from linoleate (17).

The major fatty acid compositions of plasma cholesteryl esters from WKY and SHR strains are shown in Figure 4B. Plasma cholesteryl esters contained relatively more arachidonate than did hepatic cholesteryl esters possibly

as a consequence of the LCAT reaction. The HCD feeding induced changes in the proportions of fatty acids qualitatively similar to those seen in hepatic cholesteryl esters; an increase in monoenoic fatty acids and a decrease in arachidonate were observed in both strains. Quantitatively, however, these changes were significantly less in SHR than in WKY. The fatty acid compositions of plasma lipids of SHRSP were similar to those of WKY (data not shown).

The molecular species of cholesteryl esters are thought to be produced mainly through the actions of LCAT in plasma and of ACAT in liver and intestine. Plasma LCAT and hepatic ACAT are considered to be particularly important in determining the molecular species of plasma cholesteryl esters in fasted rats. In the ND groups, the ACAT and LCAT activities were roughly similar in WKY and SHR. HCD-feeding increased the ACAT activities fivefold in WKY, and threefold in SHR. In contrast, different responses of LCAT activities to HCD-feeding were observed; LCAT activity significantly decreased to 60% in WKY, and increased in SHR (Table 1). These differences in the responses of ACAT and LCAT to the dietary changes appear to be consistent with the differences in the fatty acid patterns of plasma cholesteryl esters in WKY and SHR, because arachidonate is considered to be esterified preferentially *via* LCAT (32,36-39). The HCD-induced changes in the linoleate/arachidonate ratios of plasma and hepatic PL and cholesteryl esters (ChoE) were compared in the three strains (Fig. 5).

The increases in the linoleate/arachidonate ratios induced by HCD-feeding were seen in all strains, particularly in plasma and hepatic cholesteryl ester fractions, although the increase in the linoleate/arachidonate ratio of plasma cholesteryl esters was significantly less in SHR than in the other two strains.

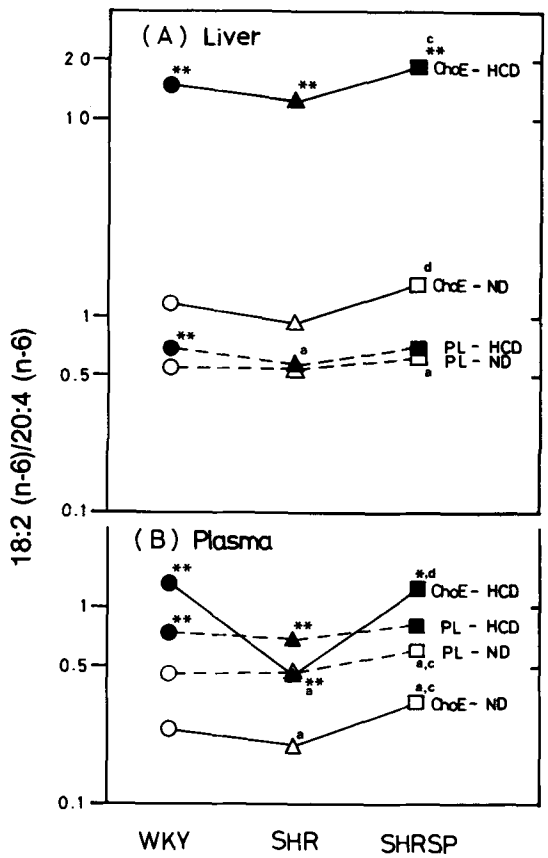

FIG. 5. Effect of a hypercholesterolemic diet on linoleate (18:2)/arachidonate (20:4) ratios of cholesterol esters and phospholipid in plasma and liver. Open symbols and closed symbols indicate the normal diet group (ND) and the hypercholesterolemic diet group (HCD), respectively. Averages of determinations (±SD) for three rats are presented. Statistical significance is shown as ** ($P < 0.01$) or * ($P < 0.05$) for the comparison of ND and HCD groups, a ($P < 0.01$) or b ($P < 0.05$) for the comparison of WKY and other strains, and c ($P < 0.01$) or d ($P < 0.05$) for the comparison of SHR and SHRSP. Abbreviations as in Figure 1.

DISCUSSION

SHR derived from the Wistar strain (WKY) are widely used as a model of hypertension. The SHRSP derived from SHR (29) have the highest blood pressure among rat strains and die mostly of cerebral bleeding (30). Some groups have reported that SHR develop hypercholesterolemia more easily than conventional strains when fed atherogenic diets (13,31); moreover, ring-like fat depositions were noted in SHR and SHRSP strains, but not in the normotensive WKY strain (15,16). However, the results obtained in the present experiments, as well as those of our previous study (17), are inconsistent with the results reported by others. Different methods used in different laboratories for the assay of cholesterol may be responsible for some of the inconsistent results. The plasma cholesterol and aortic cholesterol levels were the highest in WKY among the three strains fed the HCD. In contrast, the hepatic cholesterol contents were higher in SHR and SHRSP than in WKY. Therefore, there may be differences among WKY, SHR and SHRSP: (i) in the synthesis of lipoproteins and secretion from liver; (ii) in the synthesis of bile acid; and/or (iii) in the re-uptake of lipoproteins into liver. Although the enzymatic basis for inducing these differences in cholesterol metabolism remains to be elucidated, we conclude that hypertension does not stimulate hypercholesterolemia or fat deposition in the aorta induced by atherogenic diets.

The fatty acid compositions of hepatic and plasma cholesteryl esters changed substantially when rats were fed HCD. The increases in the proportions of oleate and in the linoleate/arachidonate ratios are interpreted to be due to elevated Δ9 desaturase activity to form oleate (32,33) and decreased elongation-desaturation activity (33). Although these changes in hepatic lipids were common to all the strains examined, the changes in plasma cholesteryl esters were significantly less in SHR than in WKY and SHRSP (Figs. 4 and 5). ACAT is known to be relatively specific for monoenoic, saturated and dienoic acyl-CoA (34-36), while LCAT is specific for arachidonate and linoleate, which are enriched in the 2-position of phosphatidylcholine (32,36-39). The relatively lower linoleate/arachidonate ratio of plasma cholesteryl esters observed in SHR, but not in WKY or SHRSP (Fig. 5), may be attributed to the difference in the relative activities of ACAT and LCAT, because the proportions of arachidonate in plasma phosphatidylcholine were very small among the three strains (Fig. 5, and data not shown). In fact, the LCAT/ACAT activity ratio was significantly lower in WKY than in SHR fed HCD diets, which could account

for part of the differences observed in the linoleate/arachidonate ratios of plasma cholesteryl esters in WKY and SHR.

Although SHRSP and SHR were derived from WKY, and the blood pressure decreases in the order of SHRSP > SHR > WKY, neither plasma lipid levels nor HCD-induced hyperlipidemia, nor aortic lipid deposition, nor the linoleate/arachidonate ratio of plasma cholesteryl esters paralleled the blood pressures measured in the three strains. Our data suggest that in rats hypertension *per se* does not promote the development of hyperlipemia or arterial fat deposition induced by the atherogenic diet.

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The Relationship Between Fatty Acid Peroxidation and α-Tocopherol Consumption in Isolated Normal and Transformed Hepatocytes

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The response of normal and transformed rat hepatocytes to oxidative stress was investigated. Isolated normal rat hepatocytes and differentiated hepatoma cells (the Fao cell line was derived from the Reuber H 35 rat hepatoma) in suspension were incubated with the ADP/Fe^{3+} chelate for 30 min at 37°C. Membrane lipid oxidation was assessed by measuring (i) free malondialdehyde (MDA) production by a high-performance liquid chromatography (HPLC) procedure, (ii) membrane fatty acid disappearance as judged by capillary gas chromatography, and (iii) α-tocopherol oxidation as determined by HPLC and electrochemical detection. The addition of iron led to increased MDA production in normal as well as in transformed cells, and to simultaneous consumption of polyunsaturated fatty acids (PUFA) and α-tocopherol. In addition, in Fao cells more α-tocopherol was consumed during lipid peroxidation while less PUFA was oxidized. Lipid peroxidation was lower in tumoral hepatocytes than in normal cells. This could be due to a difference in membrane lipid composition because of a lower PUFA content and a higher α-tocopherol level in Fao cells. During oxidation, Fao cells produced 1.5 to 2 times less MDA than normal cells, while in the tumoral cells the amount of oxidized PUFA having 3 or more double bonds was 7 to 8 times lower. Therefore, measuring MDA alone as an index of lipid peroxidation did not allow for proper comparison of the membrane lipid oxidizability of transformed cells *vs.* the membrane lipid oxidizability of normal cells.
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Several disorders, including idiopathic hemochromatosis, can lead to excess iron deposition in tissues, especially in the liver (1,2). Excess hepatic iron causes cellular injury with resultant fibrosis and cirrhosis (3). In addition, chronic iron overloading causes an increased risk of hepatocellular carcinoma (4). The pathophysiological mechanisms of hepatocellular injury due to iron overloading are unknown. Free radical iron-induced oxidative stress resulting in membrane lipid peroxidation is a potential mechanism of cellular injury during an iron overload (5-8). *In vitro* studies have shown that iron can enter both normal and transformed hepatocytes (9). Generally, tumoral cells are highly resistant to lipid peroxidation, and their proliferation index correlates directly with their resistance to lipid peroxidation inducers (10). One of the possible reasons for the low level of lipid peroxidation is a decrease in unsaturated fatty acids, the main targets of lipid oxidation and an increase in lipid antioxidant content, particularly with regard to vitamin E (11,12). Lipid peroxidation is mainly estimated by measuring malondialdehyde (MDA), a by-product of polyunsaturated fatty acid (PUFA) peroxidation (13,14).

The aim of this study was to compare the response of normal rat hepatocytes and of a Fao cell line to oxidative stress induced by iron. Fao cells are derived from the Reuber H 35 rat hepatoma and are characterized by a slow rate of growth (15). They express most of the liver-specific functions. Lipid peroxidation was estimated by free MDA production. Furthermore, the disappearance of individual membrane fatty acids was followed as was simultaneous α-tocopherol oxidation.

MATERIALS AND METHODS

Reagents. Fatty acid methyl ester (FAME) standards and 1,1,3,3-tetramethoxypropane were purchased from the Sigma Chemical Company (St. Louis, MO). α-Tocopherol, tocol, sodium dodecyl sulfate (SDS) and butylated hydroxytoluene (BHT) were supplied by the Merck Chemical Company (Darmstadt, Germany) and adenosine diphosphate (ADP) by Boehringer Mannheim (Mannheim, Germany). Ferric sulfate, disodium hydrogen phosphate, chloroform, ethanol, acetic acid and methanol were obtained from Carlo Erba (Milan, Italy). *n*-Heptane [high-performance liquid chromatography (HPLC) grade] was provided by Rathburn Chemicals (Walkerburn, Scotland).

Isolation of hepatocytes. Normal rat hepatocytes were isolated from two-month-old Sprague-Dawley male adult rats by cannulating the portal vein and perfusing the liver with a collagenase solution, as previously described (16). The cells were collected in Leibovitz medium containing 1 mg bovine serum albumin and 5 μg bovine insulin per mL. The cell suspension was filtered through gauze and allowed to sediment for 20 min in order to eliminate cell debris, blood and sinusoïdal cells, and the cells were washed three times by centrifugation at 150 × *g*, and counted. The hepatocytes were then suspended for 15 min in a mixture composed of Eagle's minimum essential medium (75%) and medium 199 (25%) with Hank's salts; the mixture contained (per mL) kanamycin (50 μg), streptomycin (50 μg), penicillin (7.5 IU), bovine insulin (5 μg), bovine serum albumin (1 mg) and $NaHCO_3$ (2.2 mg). Viability was at least 90% as determined by trypan blue exclusion.

Fao cells were plated in 75-cm^2 Nunclon® flasks. The cells were grown at 37°C to confluency using the same medium as above but supplemented with fetal calf serum (10%).

Just before the experiment, the culture medium was discarded, and Fao cells were collected from the culture and suspended in phosphate buffer (0.01 M, pH 7.45). Similarly, in normal hepatocyte suspensions, the culture medium was replaced by the same phosphate buffer.

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Abbreviations: ADP, adenosine diphosphate; BHT, butylated hydroxytoluene; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; SDS, sodium dodecyl sulfate.

For experimental purposes, each cell suspension (30×10^6 hepatocytes or Fao cells) was maintained in the presence of an ADP/Fe^{3+} system in order to obtain final 20 mM ADP and 85 μM or 170 μM iron concentrations. Each sample with ADP/Fe^{3+} was compared to control suspensions without supplementation. Samples and controls were then incubated at 37°C for 30 min.

Estimation of free MDA. Lipid peroxidation was estimated by an increase in free MDA which was measured by HPLC according to an previously described method (17). The HPLC system (LDC, Milton Roy, Riviera Beach, FL) was equipped with a Spherogel-TSK G 1000 PW size exclusion column measuring 7.5 mm i.d. × 30 cm (Cluzeau, France). The eluant was composed of 0.1 M disodium phosphate buffer, pH 8, and was run at a flow rate of 1 mL/min at ambient temperature. Absorbance was monitored at 267 nm, and sensitivity was set at 0.05 AUFS. Samples were injected using an automatic injector (LDC Promis, Milton Roy) set at a volume of 250 μL. The data were recorded and integrated with a CI 3000 LDC integrator.

The free MDA standard solution was prepared by hydrolysis of 5 μL of 1,1,3,3-tetramethoxypropane in 5 mL of 0.1 N HCl for 5 min in a boiling water-bath. A one-to-thousand dilution of the solution was prepared in 0.01 M phosphate buffer, pH 7.45 corresponding to a 6 μM MDA solution. The MDA concentration in samples was calculated based on a standard free-MDA curve.

After the hepatocytes had been incubated with iron, the cells were lysed at 0°C using an ultrasonic homogenizer. An aliquot was filtered through a 500-dalton membrane ultrafilter (Millipore, Saint Quentin, France) in a 10-mL (Amicon, Lexington, MA) cell pressurized to four bars with nitrogen gas. Two-hundred fifty μL of the ultrafiltrate obtained was injected into the HPLC system. An aliquot of the lysed solution was used to determine protein content according to Bradford (18) using the Bio-Rad (Paris, France) protein assay reagents and bovine serum albumin as standard. A Cobasbio (Roche, Neuilly Seine, France) automatic analyzer was utilized for this purpose.

Lipid analysis. An aliquot of the lysed hepatocyte suspension was used for the extraction of lipids by the SDS method (19). Lipids were extracted with a mixture of heptane, ethanol and an aqueous SDS solution with a concentration of 100 μM (2:2:1, by vol). The upper layer (heptane) containing the lipids was used for fatty acid (FA) and α-tocopherol measurements.

Fatty acids were analyzed by capillary gas chromatography (GC) of the methyl esters. The transesterification reaction was carried out according to Christie (20). An aliquot of the lipid extract, which contained heptadecanoic acid as internal standard, was evaporated to dryness and redissolved in anhydrous methanolic 0.5 M sodium methoxide. The mixture was reflux-heated to 50°C for 20 min. FAME were then extracted and redissolved in ethanol.

FAME were analyzed on a Packard 439 GC instrument (Palo Alto, CA) equipped with a flame ionization detector using the on-column injection mode. The capillary column was a Carbowax 20 M (25 m × 0.25 mm i.d.) (Cluzeau, France). The carrier gas was hydrogen at a flow rate of 2 mL/min. The temperature was programmed from 60° to 214°C at 6°C/min for 20 min, 0°C/min for 10 min, 4°C/min for 10 min, 0°C/min for 5 min, then 4°C/min to 214°C. Samples of 0.3 μL of the ethanolic FAME solution were directly injected into the chromatograph. The FAME concentration in samples was calculated using standard curves obtained on standard solutions.

α-Tocopherol was measured by HPLC using electrochemical detection. An aliquot of the lipid extract, containing tocol (500 ng/mL) as an internal standard, was evaporated to dryness, redissolved in the mobile phase and injected into the HPLC instrument (LDC, Milton Roy) equipped with a 250 mm × 4.3 mm ODS C_{18} (5 μm) column (Sopares, France). The mobile phase consisted of methanol and a 6% aqueous potassium nitrate solution (2 g/L). The flow rate was 0.8 mL/min; α-tocopherol was detected with an electrochemical detector (ESA, Coulochem model 5100 A, Sopares, France) set at +0.4 V.

Statistics. All experiments were performed in triplicate. Results are expressed as mean ± SEM, and statistical differences were analyzed by Student's *t*-test.

RESULTS

Examination of fatty acids before and after lipid peroxidation. Before lipid peroxidation was induced, the membrane FA composition of normal hepatocytes and Fao cells were measured (Table 1). The FA profiles of normal rat hepatocytes were similar to those reported previously (21,22). A high percentage (14%) of 22:6 was found in our study, but variable values are reported in the literature (23). Fao cells showed a pronounced decrease (64%) in FA content, and a parallel decrease (70%) in PUFA level. The FA distribution of Fao cells was different from that of normal hepatocytes. The percentages of saturated FA, 16:0 and 18:0, were decreased, whereas 18:1 showed a 4-fold increase. A change in PUFA distribution was also noted, except for 20:4 the percentage of which remained unchanged. The 20:2 level was greatly increased, whereas 18:2 was detected only in trace amount, and the 22:6 level was reduced by half.

After 30 min of incubation in the presence of iron, a significant PUFA loss was noted in normal hepatocytes and a lesser loss in Fao cells (Table 2). PUFA oxidation was 7 to 8 times lower in Fao cells as compared to normal hepatocytes (Table 3). The extent of PUFA oxidation increased with the degree of FA unsaturation and the iron concentration (Table 2). No appreciable oxidation was detected in saturated and unsaturated FA in both normal and tumoral hepatocytes.

Free MDA as an index of membrane lipid peroxidation. In the absence of iron, both normal and tumoral cells (Fao) exhibited a basal level of free MDA (Table 3). Induction of lipid peroxidation by chelated iron brought about an increase of free MDA, depending on the iron concentration (Table 3). Fao cells produced 1.5 to 2 times less free MDA than did normal cells under the same conditions.

The ratio between the production of MDA and the consumption of PUFA (having three or more double bonds) was 11 and 44% for normal and tumoral cells, respectively, independent of the iron concentration. The ratio was therefore not linked to the degree of lipid peroxidation (Table 3). In normal cells, values of 8–12% in regard to MDA/PUFA ratio are common (14,23). This ratio was surprisingly high (44%) in Fao cells despite a low level of n-3 PUFA which are considered the major source of MDA production in the course of lipid peroxidation (24).

Measurement of α-tocopherol before and after lipid peroxidation. Fao cells contained a higher level of α-tocopherol

TABLE 1

Fatty Acid Composition and α-Tocopherol Content of Normal Rat Hepatocytes and Fao Cells (Rat Hepatoma) Before Iron-Induced Lipid Peroxidation

	Fatty acid[a] (nmol/mg protein)	PUFA[a] (nmol/mg protein)	α-Tocopherol[a] (nmol/mg protein)	Fatty acid[a] (%)						
				16:0	18:0	18:1	18:2	20:2	20:4	22:6
Normal hepatocytes	1593 ± 128	955 ± 65	0.35 ± 0.05	19.3 ± 2.1	14.5 ± 2.7	7.6 ± 1.5	18.2 ± 1.2	2.8 ± 1.3	26.6 ± 2.1	14.7 ± 2.2
Fao cells	581 ± 52	301 ± 21	1.00 ± 0.15	11.9 ± 1.2	10.8 ± 1.12	7.7 ± 3.5	tr[b]	18.2 ± 1.9	26.2 ± 3.2	7.3 ± 1.4

[a]The results are given as the means of three independent experiments ±SEM; $P < 0.05$. PUFA, polyunsaturated fatty acids. [b]Trace amount.

TABLE 2

Fatty Acid and α-Tocopherol Oxidation During Iron-Induced Lipid Peroxidation in Normal Hepatocytes and Fao Cells[a]

Iron (μM)		PUFA (%)[b] disappearance	α-Tocopherol (%)[b] disappearance	FA (%)[b] disappearance					
				16:0	18:0	18:1	18:2	20:2	20:4	22:6
85	N[c]	26.4 ± 3.3	15.9 ± 1.3	N.D.[c]	N.D.[c]	3.6 ± 0.9	18.8 ± 3.4	34.2 ± 3.5	28.9 ± 4.2	30.9 ± 3.8
	T[c]	5.8 ± 1.2	47.3 ± 3.6	N.D.[c]	N.D.[c]	N.D.[c]	tr[c]	1.4 ± 0.5	10.5 ± 3.6	N.D.[c]
170	N[c]	36.6 ± 5.4	40.8 ± 4.9	1.2 ± 0.2	1.9 ± 0.4	5.1 ± 1.4	34.7 ± 4.2	43.4 ± 4.8	45.9 ± 5.2	40.6 ± 4.3
	T[c]	24.5 ± 3.3	55.2 ± 6.1	N.D.[c]	N.D.[c]	4.4 ± 1.2	tr[c]	25.3 ± 0.4	20.5 ± 0.4	37.4 ± 5.6

[a]Normal and transformed hepatocytes were incubated for 10 min at 37°C in the presence of ADP/Fe^{3+} (for details see Materials and Methods). Numbers for fatty acid (FA) (%) disappearance indicate the oxidized FA proportion as percentage relative to each FA.

[b]The results are given as the means of three independent experiments ± SEM; $P < 0.05$; PUFA, polyunsaturated fatty acids.

[c]Abbreviations used: N, normal hepatocytes; T, transformed hepatocytes (Fao cells); tr, trace amount; N.D., not detectable.

than did normal hepatocytes. Most workers have observed an increase in α-tocopherol content in tumor cells (25,26). The α-tocopherol/PUFA ratio was 10 times higher in Fao cells (1/300) than in normal hepatocytes (1/3000). PUFA peroxidation was accompanied by a rapid decrease in α-tocopherol content, especially in Fao cells which showed a disappearance of α-tocopherol of about 50% after 30 min of lipid peroxidation induced by both concentrations of iron (Table 2). In normal cells, α-tocopherol oxidation was lower than in Fao cells and increased with iron concentrations (Table 2).

DISCUSSION

The susceptibility to iron-induced lipid peroxidation in normal and transformed (Fao cell line) rat hepatocytes was investigated. Isolated hepatocytes in suspension proved to be a useful model for the study of cellular response to oxidative stress because the model allowed us to look into various events which influence lipid peroxidation, especially in regard to cell defense. *In vitro* studies of iron-induced lipid peroxidation have employed various iron complexes (27,28). In the present work the ADP/Fe^{3+} system was used because it is one of the iron complexes that most readily enters cells (29,30).

It is clear from the results reported in this study that there are marked differences in response to iron-induced lipid peroxidation between normal and transformed hepatocytes. Two indices of lipid peroxidation were used: oxidation of PUFA and free MDA production, the latter of which has been reported to be a more accurate index of lipid peroxidation than is the thiobarbituric acid reactive substance test (31). Yet, iron chelates catalyze the breakdown of PUFA hydroperoxides having three or more double bonds (14) and can also catalyze the degradation of amino acids, sugars and DNA to yield MDA (32). This latter oxidative degradation is not inhibited by antioxidants (33). Morel *et al.* (17) recently reported that, in the presence of iron, free MDA was completely reduced by the addition of α-tocopherol, a lipid antioxidant. Therefore, it is likely that under experimental conditions, free MDA resulted mainly from PUFA hydroperoxide breakdown.

Both lipid peroxidation indices were lowered in the Fao cells which exhibited greater resistance to lipid peroxidation compared to normal hepatocytes. Using the MDA index, lipid peroxidation could be estimated as 1.5 to 2 times lower in Fao cells. Using the other index, MDA-yielding oxidized PUFA, lipid peroxidation was 7 to 8 times lower in tumor cells. The MDA/oxidized PUFA ratio was abnormally high (44%) in Fao cells as compared to normal hepatocytes (11%). This difference may be explained by an increase in free MDA levels which could be due to: (i) changes in the breakdown pathways of lipid peroxides in Fao cells; the MDA production pathway may theoretically be increased compared to other aldehydic pathways; (ii) bound MDA to cellular materials, such as proteins (34), may possibly be decreased and the free MDA fraction measured in this study would increase; (iii) MDA metabolism may be reduced due to a decrease or a lack of activity in aldehyde dehydrogenases in tumoral cells (35). Thus, MDA as a marker of lipid peroxidation may lead to an overestimation of tumoral cell oxidizability.

Resistance to oxidative stress is a common feature of many tumoral cells (36–38). Among possible mechanisms

TABLE 3

Free MDA Production and PUFA[a] Disappearance in Normal and Tumoral Cells During Iron-Induced Lipid Peroxidation

Iron (μM)	Free MDA[b] (nmol/mg protein)		PUFA[a] disappearance[b] (nmol/mg protein)		MDA:PUFA[a] disappearance (%)	
	N[c]	T[c]	N[c]	T[c]	N[c]	T[c]
0	2.4 ± 0.1	1.0 ± 0.1	—	—	—	—
85	17.9 ± 1.7	8.4 ± 1.9	163 ± 18	19 ± 2	11	44
170	29.2 ± 2.5	21.0 ± 1.4	324 ± 24	48 ± 5	9	44

[a] Polyunsaturated fatty acids (PUFA) having three or more double bonds.

[b] The results are given as the means of three independent experiments ±SEM; $P < 0.05$; MDA, malondialdehyde.

[c] Abbreviations used: N, normal hepatocytes; T, transformed hepatocytes.

underlying this resistance, changes in lipid composition may be responsible; *i.e.*, a decrease in total lipid containing PUFA (39,40) and an increase in both cholesterol (41) and α-tocopherol (42), both of which stabilize lipid membranes. In addition, a rise in the level of cytosolic antioxidants and a reduction of cytochrome P450 activity (43) in the lipid peroxidation initiation phase were observed.

Fao cells showed similar changes: a decrease of about 65% in total FA content, including saturated and polyunsaturated fatty acids. An alteration in PUFA distribution was also observed. Also a net increase in the percentage of 18:1 was noted. Balasubramanian *et al.* (44,45) reported that oleic acid can assume the properties of an antioxidant. Moreover, no linoleic acid was detected in Fao cells, which could be related to the maintenance of Fao cells for a long time in culture. Pepin *et al.* (46) noted that normal hepatocytes, whenever cultured in a medium depleted in essential fatty acids, showed a rapid decline in linoleic acid content. Das *et al.* (47) also reported that tumor cells incorporated less linoleic acid than did normal cells in the cultured state.

Increased α-tocopherol levels are considered to be characteristics of tumor cells which may contribute to the resistance to oxidative stress. Yet some workers (48) have suggested that increased α-tocopherol may be associated with cell division. A high content of α-tocopherol, a lipid antioxidant, was also observed in Fao cells.

The oxidative stress triggered by iron caused a higher α-tocopherol consumption in Fao cells than in normal hepatocytes. The relationship between unsaturated fatty acids and α-tocopherol in membranes is complex (49). It is generally believed that α-tocopherol inhibits lipid peroxidation in biological membranes by scavenging the chain-propagating peroxyl radicals (50). Experiments have suggested that one mole of α-tocopherol completely protected *ca.* 220 moles of PUFA from autoxidation within microsomal fractions isolated from rat heart and lung (49). In our experiments, the α-tocopherol/PUFA ratio was 1/300 and 1/3000 in Fao cells and in normal hepatocytes, respectively. The high value for Fao cells could explain why PUFA oxidation was 7 to 8 times lower than in normal cells.

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Reduction of Fatty Acid Hydroperoxides by Human Parotid Saliva

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Arachidonic acid hydroperoxide (15-hydroperoxyeicosatetraenoic acid; 15-HPETE) was introduced into human parotid saliva and incubated at 37°C. Straight phase high-performance liquid chromatography analysis of the reaction mixture showed that 15-HPETE was detoxified to its reduced form, 15-hydroxyeicosatetraenoic acid, in the presence of glutathione. Therefore, it is concluded that human parotid saliva possesses fatty acid hydroperoxide-reducing ability. However, its effectiveness was found to be lower than that of blood plasma.

Lipids 28, 121–124 (1993).

Unsaturated fatty acids in foods are susceptible to enzymatic and nonenzymatic lipid peroxidation, resulting in a wide variety of oxidation products (1). Dietary intake of fatty acid hydroperoxides, primary products of lipid peroxidation, is of much concern because they are a potential source of free radicals leading to oxidative damage in the digestive tract and other tissues (2,3). Several studies on rodents have shown that dietary lipid hydroperoxides are largely metabolized before reaching the lymphatic circulation (4,5). It has also been implied that the small intestine possesses glutathione peroxidase activity to detoxify fatty acid hydroperoxides (6–8). However, little is known about the fate of dietary fatty acid hydroperoxides in the digestive tract.

Saliva is one of the digestive juices excreted from the digestive tract. Considerable amounts of saliva are excreted from the parotid, submandibular, sublingual and the large number of minor salivary glands (600 mL/day/person) (9). In an earlier paper (10), we reported that human parotid saliva has antioxidant effects on lipid peroxidation of liposomal phospholipids and fish meat. This prompted us to investigate whether human parotid saliva possesses the ability to detoxify dietary lipid hydroperoxides. We examined the behavior of arachidonic acid hydroperoxides in this extracellular fluid with or without the addition of a reducing agent. The results demonstrate that human parotid saliva possesses glutathione (GSH)-dependent fatty acid hydroperoxide-reducing ability.

MATERIALS AND METHODS

Materials. Arachidonic acid (99% grade) was obtained from Sigma Chemical Co. (St. Louis, MO). Reduced GSH and ascorbic acid were from Wako Pure Chemicals (Osaka, Japan). Oxidized glutathione (GSSG) and cysteine were provided by Nakarai Chemicals (Kyoto, Japan). α-Tocopherol was kindly supplied by Eisai Co. (Tokyo, Japan). 15-Hydroperoxyeicosa-5,8,11,13-tetraenoic acid (15-HPETE) was prepared by enzymatic oxidation of arachidonic acid using soybean lipoxygenase (Sigma Chemical Co.; Type I) (11). 15-Hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE) was obtained by reducing 15-HPETE with $NaBH_4$ (12). The solvents for chromatography were of high-performance liquid chromatography (HPLC) grade and obtained from Nakarai Chemicals. Distilled water was further purified by the Mili-Q reagent system (Millipore Corp., Bedford, MA). The other reagents were of analytical grade and used without purification.

Preparation of human saliva and blood plasma. Parotid saliva was collected from healthy adult volunteers, the salivary flow being stimulated with citric acid troches before collecting directly with a Lashery's device (13). The collected saliva was immediately used for the experiments. Blood plasma was obtained from the same volunteers (12).

Incubation of 15-HPETE in saliva. A methanol solution of 15-HPETE (10–40 nmol in 10 μL) was added to 0.2 mL of the fluid and shaken in a water bath at 37°C. After incubation, total lipids were extracted from the fluid using chloroform and methanol (12).

HPLC analysis. HPLC was run on a YMC-packed A312 SIL column (6 × 150 mm, Yamamura Kagaku, Kyoto, Japan) with a Tosoh liquid chromatograph CCPD. A Shimadzu SPD-2A variable wavelength ultraviolet detector was used to monitor the effluent at 235 nm (0.01 AUFS). The lipid extracts were evaporated *in vacuo* and then redissolved in the eluting solvent. A Reodyne 7135 loop injector (20 μL) was used to inject the sample solution onto the column. The column was then developed with a mixture of hexane/isopropanol/acetic acid (97.3:2.5:0.2, by vol) at the flow rate of 1.0 mL/min.

Other procedures. To separate the high molecular weight (HMW) fraction (molecular weight > 5000) and the low molecular weight (LMW) fraction (molecular weight < 5000) of the parotid saliva, a Sephadex G25 column (PD-10, Pharmacia Co., Uppsala, Sweden) was equilibrated with 0.01 M Tris-HCl buffer (pH 7.4). Freshly collected saliva (10 mL) was applied to the column and eluted with the same buffer. The first eluate (1.5 mL) and second eluate (1.5 mL) were collected as an HMW fraction and an LMW fraction, respectively. Simulated gastric juice was prepared by diluting a solution of conc. HCl (0.82 mL, 12M) and 0.411 g NaCl in double distilled water to a total volume of 100 mL (pH 1.22) (14).

RESULTS

Figure 1 shows the results of straight phase HPLC of 15-HPETE, 15-HETE and the lipid extracts after incubation of 15-HPETE for 30 min with the saliva or normal saline. 15-HPETE and its reduced derivative, 15-HETE, were eluted separately in the chromatogram. The efficiency of extraction of 15-HPETE and 15-HETE from normal saline was reported to be nearly 100% (12). This analytical technique was therefore used to evaluate the conversion of 15-HPETE to 15-HETE in parotid saliva and in normal saline. 15-HETE appeared in the chromatograms of the extract after incubation of 15-HPETE in the saliva. The addition of GSH (200 μM) to the saliva significantly increased the level of 15-HETE, indicating

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Abbreviations: GSH, glutathione; GSSG, oxidized form of glutathione; HMW, high molecular weight; 15-HPETE, 15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid; 15-HETE, 15-hydroxyeicosa-5,8,11,13-tetraenoic acid; HPLC, high-performance liquid chromatography; LMW, low molecular weight.

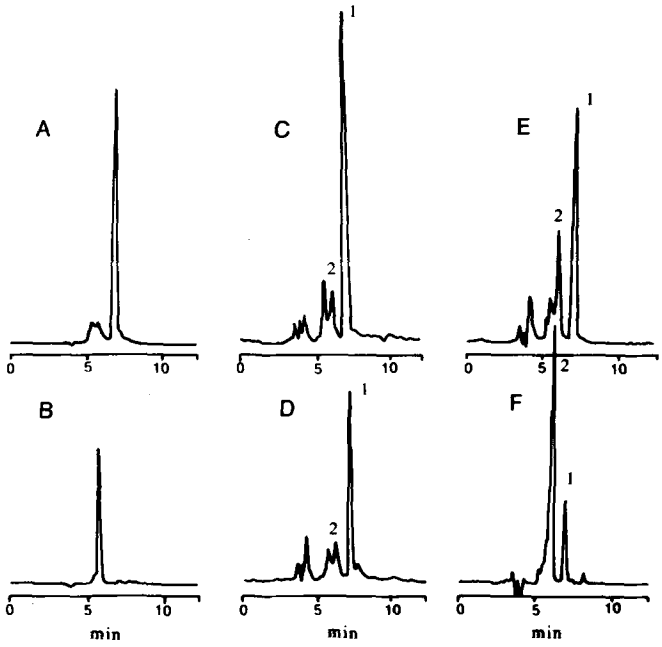

FIG. 1. High-performance liquid chromatography of 15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid (15-HPETE) after incubation with normal saline or human parotid saliva. 15-HPETE (40 nmol) was added to normal saline or parotid saliva (0.2 mL) and incubated with or without glutathione (GSH) (200 μM) at 37°C for 30 min; (A), 15-HPETE standard; (B), 15-hydroxyeicosa-5,8,11,13-tetraenoic acid (15-HETE) standard; (C), the extract from normal saline without GSH; (D), the extract from normal saline with GSH; (E), the extract from parotid saliva without GSH; (F), the extract from parotid saliva with GSH. Peak 1 and peak 2 were identified as 15-HPETE and 15-HETE, respectively.

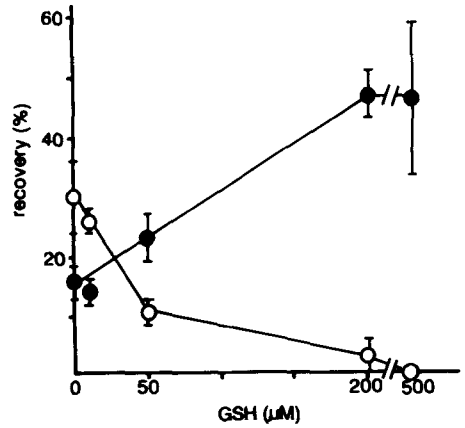

FIG. 2. Effect of GSH concentration on the reduction of 15-HPETE in parotid saliva. The reaction mixture containing 15-HPETE (10 nmol) in the saliva (0.2 mL) was incubated for 30 min at 37°C; ○, 15-HPETE; ●, 15-HETE. Abbreviations as in Figure 1.

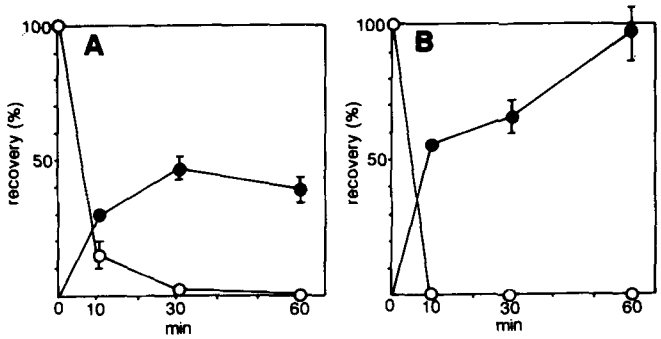

FIG. 3. Reduction of 15-HPETE to 15-HETE in parotid saliva (A) and blood plasma (B). The reaction mixture containing of 15-HPETE (10 nmol) and GSH (200 μM) in the fluid (0.2 mL) was incubated at 37°C; ○, 15-HPETE; ●, 15-HETE. Abbreviations as in Figure 1.

that GSH accelerated the conversion of 15-HPETE to 15-HETE. However, the level of 15-HETE was slightly increased after incubation of 15-HPETE with GSH in normal saline.

The effect of GSH concentration on the conversion of 15-HPETE in human saliva is shown in Figure 2. The conversion was enhanced with an increase in GSH concentration. However, the level of 15-HETE extracted from the saliva did not exceed half of the initial HPETE concentration (50 μM), even at 10 times the concentration of GSH (500 μM).

We have previously reported that human plasma has the ability to convert 15-HPETE to 15-HETE in the presence of GSH (12). Therefore, we compared the GSH-dependent reduction of 15-HPETE in the parotid saliva with that in the blood plasma from the same donor (Fig. 3). In the plasma, 15-HPETE disappeared with rapid formation of 15-HETE within the first 10-min, and all the 15-HPETE was converted to 15-HETE after 60 min. The level of 15-HETE obtained from the saliva did not exceed *ca.* 50% of the initial 15-HPETE during the incubation time. Thus, the HPETE-reducing ability of saliva was lower than that of blood plasma.

Ascorbic acid and α-tocopherol are other reducing substances of biological importance. However, the conversion was only slightly affected when GSH was replaced by these compounds (Table 1). While oxidized glutathione (GSSG) did not accelerate the formation of 15-HETE, cysteine exerted a moderate effect on the conversion of 15-HETE. The parotid saliva was separated into two fractions, an HMW fraction and an LMW fraction, by using gel-permeation chromatography. Both fractions yielded similar levels of 15-HETE after incubation for 30 min in the presence of GSH (23–27% of 15-HPETE). These levels were lower than that obtained from unseparated parotid saliva in the presence of the same concentration of GSH (45%). On the other hand, the recovery of 15-HPETE from the LMW fraction was significantly higher than that from the HMW fraction. Thus, it was apparent that the HMW fraction was responsible for the lower recovery of 15-HPETE from the parotid saliva.

Table 2 shows the effect of simulated gastric juice on the reduction of 15-HPETE in the saliva. This highly acidic juice did not accelerate the reduction regardless of the presence of GSH. However, significant conversion was observed when the juice was mixed with an equal amount of the parotid saliva. Moreover, the level of 15-HETE was higher in the mixture of saliva with simulated gastric juice than when a buffer at neutral pH was substituted for the simulated gastric juice. Thus, acidic pH, such as in the stomach environment, enhanced the hydroperoxide-reducing ability of the parotid saliva.

TABLE 1

Effect of Reducing Substances on the Recovery of 15-HPETE from Human Parotid Saliva[a]

Reducing substance	Fluid	HPETE + HETE (%)	HPETE (%)	HETE (%)
None	Saliva	23 ± 5	14 ± 3	9 ± 1
GSH	Saliva	45 ± 7	0	45 ± 7
GSH	HMW fraction	40 ± 7	12 ± 5	27 ± 2
GSH	LMW fraction	75 ± 12	55 ± 16	23 ± 2
GSSG	Saliva	31 ± 3	18 ± 3	12 ± 1
Cysteine	Saliva	25 ± 2	4 ± 3	22 ± 3
Ascorbic acid	Saliva	24 ± 4	13 ± 4	12 ± 1
α-Tocopherol	Saliva	26 ± 4	10 ± 1	11 ± 3

[a]Average value ± SD for three experiments. Reaction mixture containing 15-HPETE (10 nmol) in parotid saliva or its fraction (0.2 mL) was incubated for 30 min at 37°C. Reducing substances were added at the concentration of 200 μM. 15-HPETE, 15-hydroperoxyeicosa-5,8,11,13-tetraenoic acid; HETE, hydroxyeicosa-5,8,11,13-tetraenoic acid; GSH, glutathione; HMW, high molecular weight; LMW, low MW; GSSG, oxidized form of GSH.

TABLE 2

Effect of Simulated Gastric Juice on the Recovery of 15-HPETE[a]

System	HPETE + HETE (%)	HPETE (%)	HETE (%)
Simulated gastric juice (pH 1.2)	35 ± 9	23 ± 10	9 ± 3
Simulated gastric juice + GSH	35 ± 10	24 ± 10	7 ± 1
Simulated gastric juice + saliva + GSH	73 ± 7	21 ± 4	52 ± 3
Tris-HCl buffer (pH 7.4) + saliva + GSH	54 ± 4	21 ± 4	31 ± 4

[a]Average value ± SD for three experiments. 15-HPETE (20 nmol) was added to the stimulated gastric juice (0.2 mL) or the mixture of simulated gastric juice (0.2 mL) and parotid saliva (0.2 mL). Incubation was performed for 30 min at 37°C with or without addition of GSH (200 μM). Abbreviations as in Table 1.

DISCUSSION

Lipid hydroperoxides are mutagenic (15) and have been suspected to be carcinogenic (16). In addition, it was reported that linoleic acid hydroperoxides can induce oxidative damage in the epithelial cell of the gastrointestinal tract of rats (17). Recently, Oarada *et al.* (18) showed that dietary intake of peroxidized methyl linoleate impaired mouse lymphoid tissue, resulting in impaired immune response. Lipid hydroperoxides (LOOH) produce highly reactive alkoxyl radicals (LO•) by reacting with transition metal ions (M^{n+}) at their reduced state (19,20) (Equation 1).

$$LOOH + M^{n+} \rightarrow LO^{\cdot} + {}^{-}OH + M^{(n+1)+} \quad [1]$$

This one-electron transfer reaction may be responsible for the oxidative damage. Hydroxyl derivatives (LOH) are formed as minor products by radical combination (Equation 2) or hydrogen abstraction of alkoxyl radicals (Equation 3) (21,22).

$$H\text{-}C\text{-}O^{\cdot} + H\text{-}C\text{-}O^{\cdot} \rightarrow H\text{-}C\text{-}OH + \text{-}C{=}O \quad [2]$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \quad [3]$$

On the other hand, detoxification of lipid hydroperoxides is a nonradical two-electron transfer reaction and thus requires electron donors (AH) [IV].

$$LOOH + 2AH \rightarrow LOH + H_2O + 2A \quad [4]$$

The conversion of lipid hydroperoxides to their hydroxyl derivatives by the reaction in Equation 4 is one of the defense systems against lipid peroxidation-induced oxygen toxicity (22–26).

We have previously found that human blood plasma possesses GSH-dependent fatty acid hydroperoxide-reducing ability as a defense against excessive accumulation of lipid hydroperoxides (12). The present study also demonstrates that human parotid saliva possesses this ability to detoxify peroxidized lipids in the diets. Thiol groups seem to be required as hydrogen donors for the reaction because cysteine also enhanced the formation of hydroxyl derivatives (Table 1). Both the LMW fraction and the HMW fraction were necessary for effective conversion (Table 1).

The conversion in saliva was not as effective as the conversion in blood plasma (Fig. 3). However, the hydroperoxide-reducing ability in parotid saliva seems to be of physiological importance. It was reported that total GSH level (GSH plus GSSG) in human saliva is 0.5–1.0 μg/mL (27). Dietary foods, such as fruits and vegetables, contain considerable amounts of GSH (28,29). Jones *et al.* (29) estimated that dietary intake of GSH varies between 10- to 40-fold from 2.9 mg to 131 mg/day/person. In addition, the hydroperoxide-reducing ability of parotid saliva was found to be more effective under highly acidic conditions

(Table 2). Thus, the hydroperoxide-reducing ability of parotid saliva may be favored in the environment of the stomach.

In conclusion, human parotid saliva is able to detoxify fatty acid hydroperoxides to alcohols in the presence of GSH. This extracellular fluid thus can participate in the defense against diet-induced oxidative damage.

ACKNOWLEDGMENTS

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The Identification of the Allylic Nitrite and Nitro Derivatives of Methyl Linoleate and Methyl Linolenate by Negative Chemical Ionization Mass Spectroscopy

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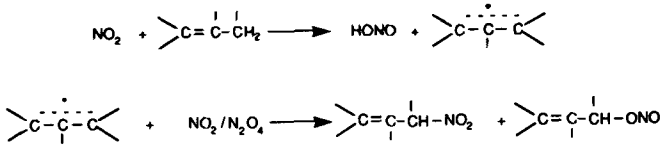
The autoxidation of polyunsaturated fatty acids is initiated both *in vivo* and *in vitro* by nitrogen dioxide. The mechanism of the initiation process is believed to involve both addition reactions and hydrogen atom abstraction reactions, with hydrogen abstraction predominating at low levels of nitrogen dioxide. Therefore low levels of nitrogen dioxide should react with polyunsaturated fatty acids to give allylic derivatives; in an anaerobic system these derivations should be allylic nitro and nitrite compounds. Using negative methane chemical ionization mass spectrometry and other analytical techniques, we have identified these allylic nitrite and nitro compounds from the reactions of low levels of nitrogen dioxide with methyl linoleate and methyl linolenate in the absence of oxygen. *Lipids 28,* **125-133 (1993).**

Nitrogen dioxide, a toxin present in polluted air (1,2), has been reported to cause pulmonary edema, pulmonary fibrosis, bronchitis (1,2) and cancer (3-6). Low levels of nitrogen dioxide have been shown to cause the initiation of the autoxidation of polyunsaturated fatty acids both *in vivo* (7-11) and *in vitro* (10,12). Nitrogen dioxide-initiated autoxidation of polyunsaturated fatty acids in cell membranes can lead to membrane damage and eventually cell death (7-10,13).

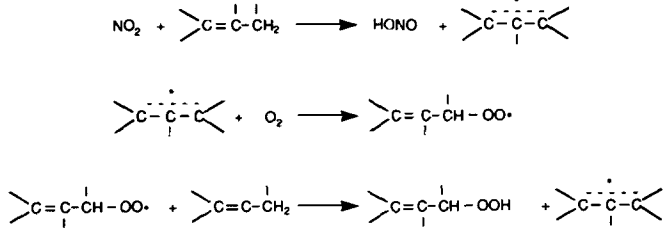
We previously reported that low levels of nitrogen dioxide initiate the autoxidation of cyclohexene by a hydrogen-atom abstraction mechanism (14,15). Recently, Postlethwait and Bidani (11) have demonstrated that nitrogen dioxide in an isolated rat lung reacts by a H-atom abstraction mechanism.

In the H-abstraction mechanism (Schemes 1 and 2), nitrogen dioxide abstracts a hydrogen atom from the allylic position of an alkene forming a resonance-stabilized radical. In the absence of oxygen (Scheme 1), nitrogen dioxide combines with the resonance-stabilized radical forming an allylic nitro or nitrite compound; in the presence of oxygen (Scheme 2), the resonance-stabilized radical combines with oxygen forming a peroxyl radical that ultimately forms allylic hydroperoxides (14). Since allylic compounds are formed *via* a H-atom abstraction mechanism, the detection of these allylic nitro or nitrite compounds can be used as a marker for nitrogen dioxide-induced H-abstraction.

In this study, we used negative chemical ionization to identify the allylic nitrite and nitro compounds formed by the reaction of nitrogen dioxide with methyl linoleate and methyl linolenate in the absence of oxygen. The allylic nitrite and nitro isomers identified are similar to the geometrical and positional isomers of methyl linoleate and methyl linolenate hydroperoxides formed in autoxidation reactions (16). Analogous to the hydroperoxide isomers, not all possible diasteriomers are produced (17). The following system is used to abbreviate the allylic nitrite (nitro) and hydroperoxide isomers: The abbreviations for two typical compounds are shown in Figure 1. The letters *c* or *t* denote a *cis* or *trans* double bond. The position of the double bond is placed before the letter *c* or *t*. The



SCHEME 1



SCHEME 2

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Abbreviations: AN, allylic nitro or nitrite compounds; *c*, *cis* double bond; DTPA, diethylenetriaminepentaacetic acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; IR, infrared; M^-, parent ion; *m/z*, mass/charge; NCI, negative chemical ionization; NMR, nuclear magnetic resonance; HP, hydroperoxide; *t*, *trans* double bond; TLC, thin-layer chromatography; UV, ultraviolet; 9*c*,11*t*,15*c*-13AN, methyl 13-nitrito-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate and methyl 13-nitro-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate; 9*t*,11*t*,15*c*-13AN, methyl 13-nitrito-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate and methyl 13-nitro-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate; 9*c*,11*t*,15*c*-13HP, methyl 13-hydrperoxy-*cis*-9,*trans*-11*cis*-15-octadecatrienoate; 9*t*,11t,15*c*-13HP, methyl 13-hydroperoxy-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate; 9*c*,12*c*,14*t*-16AN, methyl 16-nitrito-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate and methyl 16-nitro-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate; 9*c*,12*t*,14t-16AN, methyl 16-nitrito-*cis*-9,*trans*-12,*trans*-14-octadecatrienoate and methyl 16-nitro-*cis*-9,*trans*-12,*trans*-14-octadecadienoate; 9*c*,12*c*,14*t*-16HP, methyl 16-hydroperoxy-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate; 9*c*,12*t*,14*t*-16HP, methyl 16-hydroperoxy-*cis*-9,*trans*-12,*trans*-14-octadecatrienoate; 9*c*,13*t*,15*c*-12AN, methyl 12-nitrito-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate and methyl 12-nitro-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate; 9*c*,13*t*,15*t*-12AN, methyl 12-nitrito-*cis*-9,*trans*-13,*trans*-15-octadecatrienoate and methyl 12-nitro-*cis*-9,*trans*-12,*trans*-15-octadecatrienoate; 9*c*,13t,15*c*-12HP, methyl 12-hydroperoxy-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate; 9*c*,13*t*,15*t*-12HP, methyl 12-hydroperoxy-*cis*-9,*trans*-13,*trans*-15-octadecatrienoate 10*t*,12*c*,15*c*-9AN, methyl 9-nitrito-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate and methyl 9-nitro-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate; 10*t*,12t,15*c*-9AN, methyl 9-nitrito-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate and methyl 9-nitro-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate; 10*t*,12*c*,15*c*-9HP, methyl 9-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate; 10*t*,12*t*,15*c*-9HP, methyl 9-hydroperoxy-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate.

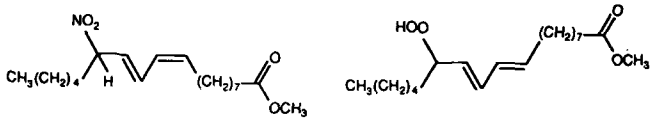


FIG. 1. Examples of the abbreviation systems used: Methyl 13-nitro-*cis*-9,*trans*-11-octadecadienoate and methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate are abbreviated as 9*c*,11*t*-13AN and 9*c*,11*t*-13HP.

letters "AN" are used to signify an allylic nitro or nitrite group and the letters "HP" denote a hydroperoxide functional group. The position of the functional group is placed before the letters AN or HP.

EXPERIMENTAL PROCEDURES

Materials. Methyl linoleate and methyl linolenate, 99% pure by gas chromatography (GC) (Sigma, St. Louis, MO), was purified by eluting the esters through four columns each of which contained 1.0 g of alumina (neutral, Aldrich, Milwaukee, WI), and the last of which also contained in the tip 0.02 g of diethylenetriaminepentaacetic acid (DTPA) to remove any trace metals. These purification steps were done in a glove bag under nitrogen.

Nitrogen dioxide was generated from dinitrogen tetroxide (99.5%, Matheson, Secaucus, NJ). Dinitrogen tetroxide was placed in a glass bulb with phosphorus pentoxide and purged of oxygen with argon by freezing and thawing the dinitrogen tetroxide three times. Isopentyl nitrite (97%, Aldrich), and nitrocyclohexane (97%, Aldrich) were used as infrared (IR) standards. Hexane, isopropanol and dichloromethane, all high-performance liquid chromatography (HPLC) grade (Mallinckrodt, St. Louis, MO), were purged of oxygen before use. Ultra pure helium (99.9999%, chromatographic grade, Air Products, Allentown, PA) was used as the carrier gas for the reaction. A thymol trap was made by dissolving 0.4 g of thymol (Sigma) and 4 g of sodium hydroxide (97%, EM Science, Gibbstown, NJ) in 1 L of deionized water.

Instrumentation. The Ultraviolet (UV) spectra were taken on a Hewlett-Packard (Avondale, PA) 8451A diode array spectrophotometer in hexane/isopropanol (99:1, vol/vol); the IR spectra were obtained on an IBM (Armonk, NY) IR/45 spectrometer. HPLC was done on a Varian (Palo Alto, CA) 5000 series instrument with a UV detector set at a 215 nm wavelength with a 16-nm slit width was used to separate the hydroperoxide and allylic nitro and nitrite isomers. A 25 × 0.46 cm silica column (Rainin, Woburn, MA; Microsorb) was used with hexane/isopropanol (99:1, vol/vol) at a flow rate of 1 mL/min. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (Billerica, MA) 200 MHz instrument using deuterated chloroform as the solvent. Negative methane chemical ionization mass spectrometry was performed on a Finnigan MAT (San Jose, CA) TSQ 4500 mass spectrometer using a direct exposure probe ramped to 1 A at a rate of 10 mA/s. Methane gas at a pressure of 0.5 Torr was used for chemical ionization. The mass range scanned in the negative mode was 44–800 amu. The source temperature was 150°C and the manifold temperature was 100°C.

Methods. The hydroperoxides of methyl linoleate listed in Table 1 were formed by exposing methyl linoleate that was kept in a clear glass vial to air for one week at room temperature. The four hydroperoxides were separated from the starting material by thin-layer chromatography (TLC) using hexane/isopropanol (94:6, vol/vol). The mixture of hydroperoxide isomers was extracted from the silica gel with dichloromethane, concentrated and resuspended in hexane. The individual positional and geometrical hydroperoxide isomers were isolated from the mixture of hydroperoxides by HPLC and analyzed by UV spectrophotometry, NMR and negative methane chemical ionization (NCl) mass spectrometry.

The eight methyl linolenate hydroperoxide isomers listed in Table 2 were formed from methyl linolenate that

TABLE 1

Methyl Linoleate Hydroperoxide and Allylic Nitrite and Nitro Isomers

Compounds
Hydroperoxides of methyl linoleate:
Methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate
Methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate
Methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate
Methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate
Allylic nitrite and nitro isomers of methyl linoleate:
Methyl 13-nitrito-*cis*-9,*trans*-11-octadecadienoate
Methyl 13-nitrito-*trans*-9,*trans*-11-octadecadienoate
Methyl 9-nitrito-*trans*-10,*cis*-12-octadecadienoate
Methyl 9-nitrito-*trans*-10,*trans*-12-octadecadienoate
Methyl 13-nitro-*cis*-9,*trans*-11-octadecadienoate
Methyl 13-nitro-*trans*-9,*trans*-11-octadecadienoate
Methyl 9-nitro-*trans*-10,*cis*-12-octadecadienoate
Methyl 9-nitro-*trans*-10,*cis*-12-octadecadienoate

TABLE 2

Methyl Linolenate Hydroperoxide Isomers and Their Abbreviations

Compounds	Abbreviations
Methyl 16-hydroperoxy-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate	9*c*,12*c*,14*t*-16HP
Methyl 16-hydroperoxy-*cis*-9,*trans*-12,*trans*-14-octadecatrienoate	9*c*,12*t*,14*t*-16HP
Methyl 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate	9*c*,11*t*,15*c*-13HP
Methyl 13-hydroperoxy-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate	9*t*,11*t*,15*c*-13HP
Methyl 12-hydroperoxy-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate	9*c*,13t,15*c*-12HP
Methyl 12-hydroperoxy-*cis*-9,*trans*-13,*trans*-15-octadecatrienoate	9*c*,13*t*,15*t*-12HP
Methyl 9-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate	10*t*,12*c*,15*c*-9HP
Methyl 9-hydroperoxy-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate	10*t*,12*t*,15*c*-9HP

was kept in a clear glass vial and exposed to air for one week at room temperature. Mixtures of all eight of the hydroperoxide isomers were isolated by TLC from the methyl linolenate starting material. The individual hydroperoxide isomers are not resolved by adsorption phase HPLC and elute from the column as four groups of unresolved positional and geometrical isomers. Each group of unresolved hydroperoxides was isolated by HPLC and analyzed by NMR.

A sodium borohydride reduction (16) was used to reduce the four groups of unresolved geometrical and positional methyl linolenate hydroperoxide isomers eluting by HPLC as four peaks (I–IV) (Fig. 2). The eight isomeric alcohols formed by reduction are resolved by HPLC. Individual isomers were identified by their HPLC elution order, as given by Chan and Levett (16); the hydroperoxide assignments are given in Table 3.

The mixtures of allylic nitrite and nitro isomers (AN) of methyl linoleate listed in Table 1 were prepared by allowing 40 ppm of nitrogen dioxide in helium to react with purified methyl linoleate (neat, 3.4 mmole) for one hour. A bubbler apparatus similar to the one utilized in our earlier study (14) was used at a carrier gas flow rate of 60 mL/min at 25°C. The entire reaction was carried out in a glove bag under nitrogen. The nitrogen dioxide concentration in the carrier gas was determined by Saltzman analysis (18) of the thymol trap solution used in a run with no fatty acid in the apparatus. The mixture of allylic nitrite and nitro compounds was separated from the methyl linoleate starting material by TLC using hexane/isopropanol (94:6, vol/vol) that had been purged of oxygen in a glove bag under an atmosphere of nitrogen. The

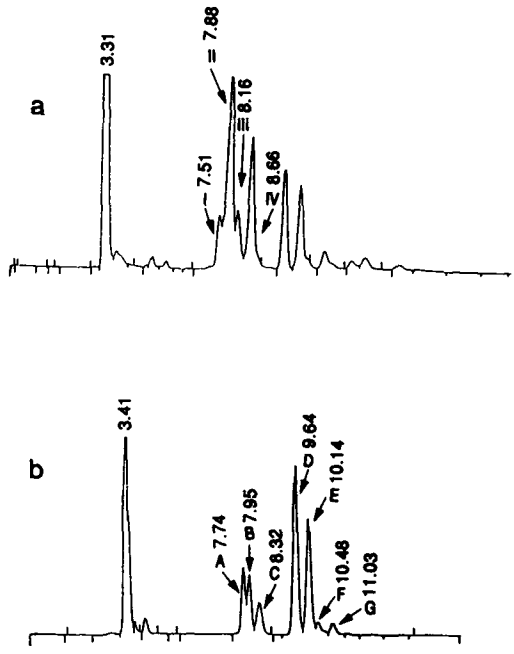


FIG. 2. High-performance liquid chromatographic chromatograms of: (a) linolenate hydroperoxide fractions that were isolated (I-IV) and (b) sodium borohydride-reduced linolenate hydroperoxides. Linolenate alcohols: A = 13-*cis,trans;* B = 12-*cis,trans;* C = 12-*trans,trans* and 13-*trans,trans;* D = 16-*cis,trans;* E = 9-*cis,trans;* F = 16-*trans,trans;* and G = 9-*trans,trans* (16).

TABLE 3

Methyl Linolenate Hydroperoxide Fraction Assignments

Fraction numbers[a]	Hydroperoxides
I	(9*c*,11*t*,15*c*-13HP)
II	(9*c*,11*t*,15*c*-13HP), (9*c*,13*t*,15*c*-12HP), (9*c*,13*t*,15*t*-12HP), (9*t*,11*t*,15*c*-13HP), and (9*c*,12*c*,14*t*-16HP)
III	(9*t*,11*t*,15*c*-13HP) and (9*c*,12*t*,14*t*-16HP)
IV	(10*t*,12*c*,15*c*-9HP) and (10*t*,12*t*,15*c*-9HP)

[a]A 25 × 0.46 cm silica column with hexane/isopropanol (99:1, vol/vol) at a flow rate of 1 mL/min was used for high-performance liquid chromatography (HPLC) analysis. The four fractions refer to the four peaks shown in the HPLC chromatogram in Figure 4b.

positional and geometrical isomers, which contained unresolved nitrite and nitro isomers, were isolated by HPLC.

The allylic nitrite and nitro isomers (AN) of methyl linolenate that were identified are listed in Table 4. These isomers were prepared by allowing purified methyl linolenate to react with 3 ppm of nitrogen dioxide in ultrapure helium for one hour. The mixture of allylic nitrite and nitro isomers was isolated from the starting material by TLC. The allylic nitrite and nitro isomers of methyl linolenate elute in HPLC as four peaks containing mixtures of unresolved positional and geometrical nitrite and nitro isomers. The four groups of unresolved isomers were analyzed by NMR and negative methane chemical ionization.

RESULTS AND DISCUSSION

Methyl linoleate allylic nitrite (nitro) and hydroperoxide isomers. The HPLC trace of the allylic nitrite (nitro) and hydroperoxide isomers of methyl linoleate are presented in Figure 3. The comparable hydroperoxide and allylic nitrite and nitro compounds elute at similar retention times.

Negative chemical ionization (NCI) mass spectrometry was used to distinguish these two types of compounds. NCI has previously been used for the analysis of various types of nitro and chlorinated compounds and organophosphates (19–24). The NCI spectra for the allylic nitrite (nitro) isomers of methyl linoleate (Table 5) display a *m/z* 46 and *m/z* 62 ion that is not detected in the NCI spectra for the hydroperoxides (Table 5). The *m/z* 46 ion is formed by dissociative electron capture and is a characteristic ion for nitro-containing compounds (21,23,24). The *m/z* 62 ion is indicative of a nitrate function (21) and is though to originate from homolysis of the O–N bond of the allylic nitrite isomers (25). The formation of the alkoxyl radical in the source of the mass spectrometer is followed by the termination of the radical with NO_2 to form the allylic nitrate. The allylic nitrate then undergoes C–O bond scission in the source of the mass spectrometer to give the *m/z* 62 ion. The *m/z* 62 ion is believed to be formed in the source rather than to originate from an allylic nitrate present in the sample, because a *m/z* 355 ion indicative of the M^- molecular ion for the allylic nitrate was not detected.

TABLE 4

Allylic Nitrite(nitro) Isomers of Methyl Linolenate and Their Abbreviations

Compounds	Abbreviations
Methyl 16-nitrito-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate Methyl 16-nitro-*cis*-9,*cis*-12,*trans*-14-octadecatrienoate	9*c*,12*c*,14*t*-16AN
Methyl 16-nitrito-*cis*-9,*trans*-12,*trans*-14-octadecatrienoate Methyl 16-nitro-*cis*-9,*trans*-12,*trans*-14-octadecatrienoate	9*c*,12*t*,14*t*-16AN
Methyl 13-nitrito-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate Methyl 13-nitro-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate	9*c*,11*t*,15*c*-13AN
Methyl 13-nitrito-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate Methyl 13-nitro-*trans*-9,*trans*-11,*cis*-15-octadecatrienoate	9*t*,11*t*,15*c*-13AN
Methyl 12-nitrito-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate Methyl 12-nitro-*cis*-9,*trans*-13,*cis*-15-octadecatrienoate	9*c*,13*t*,15*c*-12AN
Methyl 12-nitrito-*cis*-9,*trans*-13,*trans*-15-octadecatrienoate Methyl 12-nitro-*cis*-9,*trans*-13,*trans*-15-octadecatrienoate	9*c*,13*t*,15*t*-12AN
Methyl 9-nitrito-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate Methyl 9-nitro-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate	10*t*,12*c*,15*c*-9AN
Methyl 9-nitrito-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate Methyl 9-nitro-*trans*-10,*trans*-12,*cis*-15-octadecatrienoate	10*t*,12*t*,15*c*-9AN

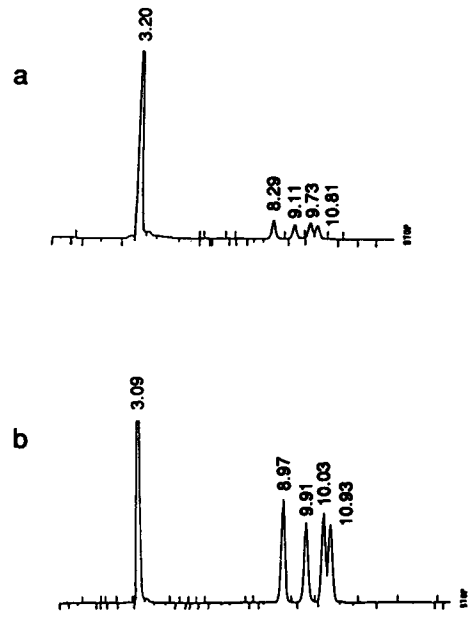


FIG. 3. High-performance liquid chromatographic chromatograms of the (a) allylic nitrite(nitro) isomers and (b) hydroperoxide isomers.

The *m/z* 339 M^- molecular ion of the allylic nitrite (nitro) isomers formed by resonance capture (23) (Table 5) reflects the ability of the compound to stabilize a negative charge (23). The *m/z* 326 molecular ion of the hydroperoxides (Table 5) was not detected. The $[M - H]^-$ ion of *m/z* 338 for the allylic nitrite (nitro) and *m/z* 325 ion for the hydroperoxides of methyl linoleate is caused by dissociative electron capture (23). The $[M + H]^-$ *m/z* 340 ion in the spectra of the allylic nitrites (nitro) is formed by H-abstraction reactions of the M^- ion (22). The Formation of $[M + H]^-$ ions also were reported by Dougherty and Dalton (22) for all chlorinated compounds that form a molecular ion. Consequently, the hydroperoxide isomers do not exhibit an M^- ion or an $[M + H]^-$ ion of *m/z* 327.

Determining the position of the hydroperoxide and nitrite functional groups. The positions of the nitrite group in the allylic nitrite isomers and of the HOO^- group in the hydroperoxide isomers were determined by the formation of an aldehyde produced from the thermal homolysis of the O-N and O-O bonds in the source of the mass spectrometer. The M^- ion of the aldehydes can be seen in the NCI spectra. Hydroperoxides have been reported to undergo thermal homolysis of the O-O bond to form an alkoxyl radical in the source of the mass spectrometer (26,27) which undergoes C-C bond cleavage to

TABLE 5

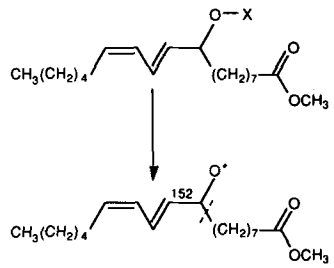
Negative Chemical Ionization Spectral Data for the Hydroperoxides and Allylic Nitrite and Nitro Compounds of Methyl Linoleate

Compounds	NO_2	NO_3	*(m/z)* 151	*(m/z)* 237	$[M - H]^-$	M^-	$[M + H]^-$
9-Hydroperoxide *(trans,trans)*	—	—	60%	—	*m/z* 325 (25%)	—	—
9-Hydroperoxide *(cis,trans)*	—	—	38%	—	*m/z* 325 (9%)	—	
9-Nitrito or (nitro) *(cis,trans)*	26%	19%	10%	—	*m/z* 338 (15%)	339 (8%)	340 (15%)
9-Nitrito or (nitro) *(trans,trans)*	78%	62%	12%	—	*m/z* 338 (21%)	339 (11%)	340 (22%)
13-Hydroperoxide *(cis,trans)*	—	—	—	53%	*m/z* 325 (9%)	—	—
13-Hydroperoxide *(trans,trans)*	—	—	—	74%	*m/z* 325 (22%	—	—
13-Nitrito or (nitro) *(cis,trans)*	72%	24%	—	25%	*m/z* 338 (20%)	339 (9%)	340 (20%)
13-Nitrito or (nitro) *(trans,trans)*	81%	40%	—	18%	*m/z* 338)12%)	339 (5%)	340 (12%)

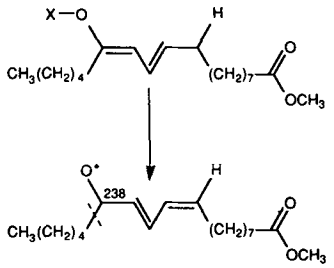
form an aldehyde (26). One of the major aldehydes reported to be formed by thermal homolysis of the 9-hydroperoxide isomers of methyl linoleate is 2,4-decadienal (26,27) (Scheme 3, where X = -OH, -NO). The major aldehyde formed by thermal homolysis of the 13-hydroperoxide of methyl linoleate is methyl 13-oxo-9,11-tridecadienoate (28,29) (Scheme 4, where X = -OH, -NO). The 9- and 13-positional allylic nitrite isomers also can form the same intermediate alkoxyl radicals that can undergo C-C bond scission to form 2,4-decadienal and methyl 13-oxo-9,11-tridecadienoate (Schemes 3 and 4).

In the NCI spectra for the 9- and 13-hydroperoxides (Table 5), a $[M - H]^-$ ion of *m/z* 151 for 2,4-decadienal is detected for the 9-hydroperoxides. Correspondingly, the $[M - H]^-$ ion of *m/z* 237 for methyl 13-oxo-9,11-tridecadienoate is seen in the NCI spectra for the 13-hydroperoxides (Table 5). The $[M - H]^-$ *m/z* 151 ion of 2,4-decadienal also is detected in the NCI spectra for the 9-allylic nitrite(nitro) isomers (Table 5). Similarly, the $[M - H]^-$ *m/z* 237 ion for methyl 13-oxo-9,11-tridecadienoate is detected in the NCI spectra for the 13-allylic nitrite(nitro) isomers. The detection of the *m/z* 151 ion and the *m/z* 237 ion in the NCI spectra of the 9- and 13-allylic nitrite(nitro) compounds, respectively, confirms the position of the functional group.

The presence of the nitro functional group. The coelution of the allylic nitro and nitrite isomers was determined by hydrolyzing the 13-*cis,trans*-allylic nitrite(nitro) compound; alkyl nitrites are known to hydrolyze to alcohols (30). The hydrolysis products of the 13-*cis,trans*-allylic nitrite(nitro) isomers display the *m/z* 46 and *m/z* 62 ions indicative of the nitro and nitrate groups by NCI. The $[M - H]^-$ *m/z* 338 and $[M + H]^-$ *m/z* 340 ions also are present. If the sample consisted of only the allylic nitrite isomer, the *m/z* 46, *m/z* 62, *m/z* 338 and *m/z* 340 ions would not have been detected in the hydrolyzed sample since alcohols do not give these ions.



SCHEME 3



SCHEME 4

IR spectral data. Table 6 displays IR data for isopentyl nitrite, nitrocyclohexane, a mixture of isopentyl nitrite and nitrocyclohexane, and our allylic nitrite(nitro) isomers. Stretching frequencies at 1603 cm^{-1} and 1587 cm^{-1} for the allylic nitrite(nitro) isomers may be caused by the presence of an oxime (31) formed by the decomposition of the allylic nitrite isomers. The stretching frequency for the nitro functional group is 1377 cm^{-1}. The stretching frequencies we observed diverge from the normal values, perhaps because of the electron-withdrawing effects of the double bonds (32).

Maximum UV absorptions. The UV maximum absorption wavelength of allylic nitrite(nitro) isomers isolated by HPLC using hexane/isopropanol (99:1, vol/vol) is 236 ± 2 nm for the *cis,trans* isomers and 234 ± 2 nm for the *trans,trans* isomers. The methyl linoleate hydroperoxides exhibit a UV maximum at 234 ± 2 nm for the *cis,trans* and 232 ± 2 nm for the *trans,trans* in hexane/isopropanol (99:1, vol/vol); these absorptions indicate that both isomers have conjugated double bonds (33).

Proton NMR data of the allylic nitrite(nitro) and hydroperoxide isomers. The proton NMR data for the 13-*cis,trans* and 13-*trans,trans* allylic nitrite(nitro) isomers are listed in Table 7. The 13-*cis,trans* and 13-*trans,trans* hydroperoxides reported by Gardner and Plattner (28) have similar chemical shifts and splitting patterns. The proton NMR data of the 9- and 13-allylic nitrite(nitro) isomers have the same chemical shifts and splitting patterns (Table 8). The 9- and 13-hydroperoxides of methyl linoleate also are reported to have identical proton NMR spectra (28). These similarities between the proton NMR data of the allylic nitrites and hydroperoxide isomers were used to confirm the structures assigned to the allylic nitrite(nitro) isomers.

NCI of the allylic nitrite(nitro) isomers of methyl linolenate. The allylic nitrite(nitro) isomers and hydroperoxides of methyl linolenate elute at similar retention times (Fig. 4), as do the allylic nitrite(nitro) isomers and hydroperoxides of methyl linoleate (Fig. 3). NCI mass spectrometry was used to identify the allylic nitrite(nitro) isomers of methyl linolenate that elute by HPLC as four groups (labeled "fraction I-IV") of mixtures of positional and geometrical isomers.

The NCI spectral data for the mixture of allylic nitrite (nitro) isomers in fraction I are shown in Table 9. The $[M + H]^-$ ion of *m/z* 338 and M^- ion of *m/z* 337 detected in this fraction confirm the molecular weight of the allylic nitrite(nitro) isomer. The *m/z* 46 ion, indicative of a nitro group, and the *m/z* 62 ion, indicative of a nitrate, also are

TABLE 6

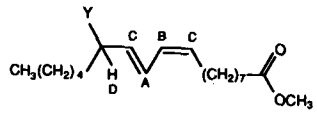
Infrared Stretching Frequencies of Nitro and Nitrite Compounds[a]

Compounds	IR frequencies (cm^{-1})
Allylic nitrite(nitro) isomers	1603, 1587, 1377
Isopentyl nitrite	1640, 1553
Nitrocylohexane	1545, 1379
Isopentyl nitrite and nitrocyclohexane	1653, 1635, 1545, 1377

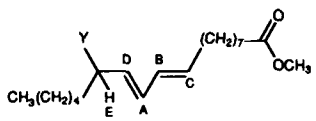
[a]The infrared (IR) spectra of the neat samples were taken with an IBM IR/45 spectrometer.

TABLE 7

Nuclear Magnetic Resonance Data of the 13-*cis,trans*- and 13-*trans,trans*-Allylic Nitrite(nitro) Compounds of Methyl Linoleate[a]



STRUCTURE 1



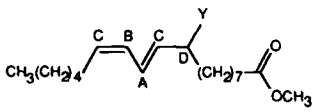
STRUCTURE 2

13-*c,t*-Allylic nitrite(nitro)	13-*t,t*-Allylic nitrite(nitro)
A = 6.58 ppm (*dd*, *J* = 15,10.5)	A = 6.29 ppm (*dd*, *J* = 15.5,10.5)
B = 6.02 ppm (*t*, *J* = 12.5,10.5)	B = 6.05 ppm (*t*, *J* = 15,12.5)
C = 5.5 ppm (*m*, *J* = 9)	C = 5.77 ppm (*dd*, *J* = 15.5,6.5)
D = 4.4 ppm (*m*, *J* = 7.5)	D = 5.47 ppm (*dd*, *J* = 15,9)
	E = 4.34 ppm (*m*, *J* = 7.5)

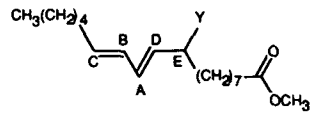
[a]*dd* = doublet doubled, *t* = triplet, *m* = multiplet; the chemical shift (mult,*J*,Hz) is given. In Structures 1 and 2, Y = NO_2, ONO.

TABLE 8

The Nuclear Magnetic Resonance Data of the 9-*cis,trans*-Allylic Nitrite(nitro) and 9-*trans,trans*-Allylic Nitrite(nitro)[a]



STRUCTURE 3



STRUCTURE 4

9-*c,t*-Allylic nitrite(nitro)	9-*t,t*-Allylic nitrite(nitro)
A = 6.58 ppm, (*dd*, *J* = 15,10.5)	A = 6.29 ppm (*dd*, *J* = 15.5,10.5)
B = 6.02 ppm, (*t*, *J* = 12.5,10.5	B = 6.05 ppm (*t*, *J* = 15,12.5)
C = 5.5 ppm, (*m*, *J* = 9)	C = 5.77 ppm (*dd*, *J* = 15.5,6.5)
D = 4.4 ppm, (*m*, *J* = 7.5)	D = 5.47 ppm (*dd*, *J* = 15,9)
	E = 4.34 ppm (*m*, *J* = 7.5)

[a]*dd* = doublet doubled, *t* = triplet, *m* = multiplet; the chemical shifts (mult/*J*, Hz) are given. In Structures 3 and 4, Y = NO_2, ONO.

TABLE 9

Negative Chemical Ionization Mass Spectral Data for the Allylic Nitrite and Nitro Compounds of Methyl Linolenate

Fractions[a]	NO_2	NO_3	(*m/z*) 109	(*m/z*) 149	(*m/z*) 237	(*m/z*) 277	M^-	$[M + H]^-$
I	72%	2%	—	—	70%	—	6%	18%
II	17%	2%	2%	—	—	37%	<1%	<1%
III	100%	2%	—	—	5%	12%	5%	10%
IV	98%	12%	—	76%	—	—	10%	20%

[a]The four fractions refer to the four peaks in the chromatogram in Figure 4a.

detected. The $[M - H]^-$ ion of methyl 13-oxotridecadienoate, *m/z* 237, is formed by O–N homolysis and subsequent carbon-carbon bond scission of the 13-allylic nitrite isomer (Scheme 5).

The NCI spectral data for the mixture of allylic nitrite (nitro) isomers in fraction II are shown in Table 9. The $[M + H]^-$ ion at *m/z* 338, the parent ion at *m/z* 337, the NO_2^- ion at *m/z* 46 and the NO_3^- ion at *m/z* 62 are observed. The *m/z* 109 ion is the $[M - H]^-$ ion of 2,4-heptadienal formed by thermal homolysis of the 12-allylic nitrite isomer(s) (Scheme 6). The *m/z* 277 ion is the $[M - H]^-$ ion of methyl 16-oxo-9,12,14-hexadecatrienoate isomer(s) formed by thermal homolysis of the 16-allylic nitrite isomer (Scheme 7).

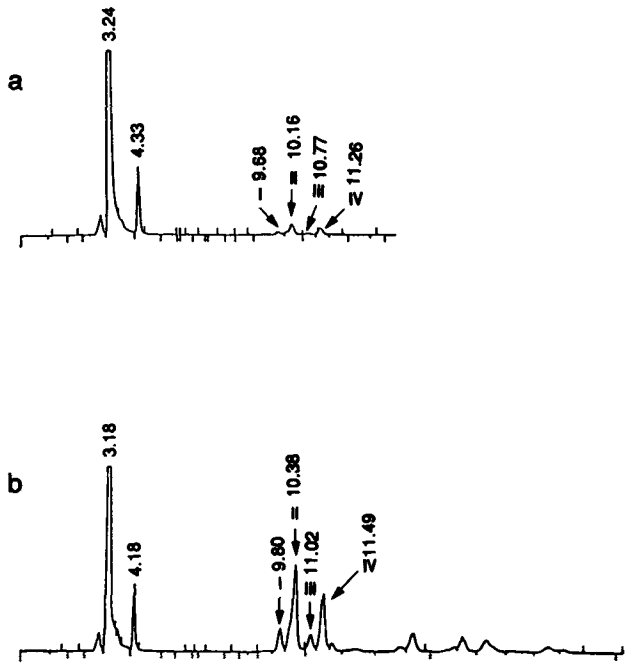
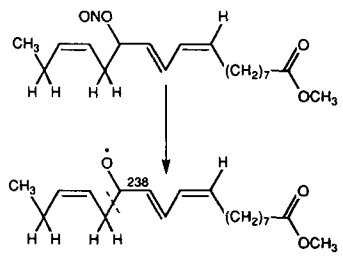
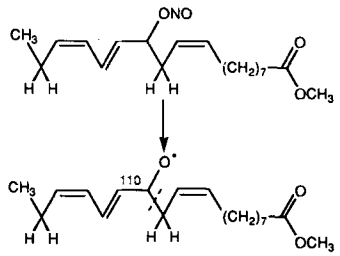


FIG. 4. High-performance liquid chromatographic chromatograms of methyl linolenate (a) allylic nitrite(nitro) isomers and (b) hydroperoxide isomers.



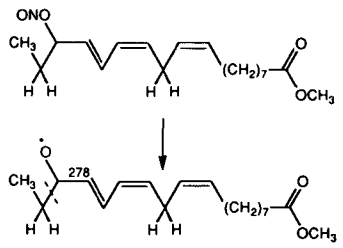
SCHEME 5



SCHEME 6

The NCI spectral data for the mixture of allylic nitrite(nitro) isomers in fraction III are shown in Table 9. The $[M + H]^-$ ion of *m/z* 338, M^- ion of *m/z* 337, NO_2^- ion *m/z* 46, and NO_3^- ion of *m/z* 62 are all observed. The *m/z* 237 ion for the 13-allylic nitrite isomer and the *m/z* 277 ion for the 16-allylic nitrite isomer were both detected.

The NCI spectral data of the mixture of allylic nitrite(nitro) isomers in fraction IV are shown in Table 9.



SCHEME 7

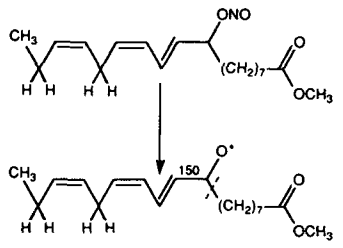
The $[M + H]^-$ ion of *m/z* 338, M^- ion of *m/z* 337, NO_2^- ion of *m/z* 46 and the NO_3^- ion of *m/z* 62 are detected. The *m/z* 149 ion is the $[M - H]^-$ ion of 2,4,7-decatrienal formed by thermal homolysis of the 9-allylic nitrite isomer (Scheme 8).

Proton NMR analysis of the hydroperoxide and allylic nitrite(nitro) fractions of methyl linolenate. The proton NMR chemical shifts and splitting patterns of the olefinic region of the hydroperoxide fractions of methyl linolenate are similar to those of the allylic nitrite(nitro) isomers (Table 10). The proton NMR of the methyl linoleate hydroperoxides and allylic nitrite(nitro) isomers also are very similar.

Assignments of the allylic nitrite(nitro) isomer fractions of methyl linolenate. The assignments of the allylic nitrite (nitro) isomers of methyl linolenate obtained from the NCI and NMR data presented above are listed in Table 11. Since the isomers of the methyl linoleate hydroperoxides and allylic nitrite isomers of methyl linoleate elute at similar retention times, the assigned *cis,trans* and *trans,trans* geometries of the allylic nitrite (nitro) isomers of methyl linolenate were based on the similar elution time profile of the hydroperoxide isomers.

We have shown that nitrogen dioxide reacts with methyl linoleate and linolenate in the absence of oxygen to give allylic nitro and nitrite isomers (34). Our data demonstrate that these isomers can be identified by NCI mass spectrometry. We have previously rationalized these products by a mechanism involving hydrogen atom abstraction by nitrogen dioxide (14,15).

The allylic nitrite(nitro) compounds and hydroperoxides elute at similar retention times by adsorption HPLC. The UV maximum absorption wavelengths and the proton NMR spectra of the allylic nitrite(nitro) and hydroperoxide compounds also are similar. These similarities were used to confirm the structure of the nitrite(nitro) compounds.



SCHEME 8

TABLE 10

Proton Nuclear Magnetic Resonance Data of the Hydroperoxide and Allylic Nitrite(nitro) Fractions of Methyl Linolenate[a]

Fraction II[b]		Fraction IV[c]	
ROOH	RNO_2/RONO	ROOH	RNO_2/RONO
6.6 ppm *(m)*	6.6 ppm *(m)*	6.6 ppm *(m)*	6.6 ppm *(m)*
6.05 ppm *(t)*	6.05 ppm *(t)*	6.05 ppm *(t)*	6.05 ppm *(t)*
5.3-5.7 ppm *(br,m)*	5.3-5.65 ppm *(br,m)*	5.3-5.65 ppm *(br,m)*	5.3-5.65 ppm *(br,m)*
4.35 ppm *(br,m)*	4.35 ppm *(m)*	4.4 ppm *(m)*	4.38 *(m)*

[a] *br* = broad, *m* = multiplet and *t* = triplet. Since the fractions are a mixture of isomers, the coupling constants are not shown.

[b] Fraction II for the hydroperoxides and allylic nitrite(nitro) isomers was isolated using a silica column with hexane/isopropanol (99:1, vol/vol) at a flow rate of 1 mL/min. The retention time for the hydroperoxide in Figure 4b was 10.38 min and for the allylic nitrite(nitro) in Figure 4a was 10.16 min.

[c] The retention time of fraction IV is 11.49 min for the hydroperoxide and 11.26 min for the allylic nitrite(nitro) isomer.

TABLE 11

Allylic Nitrite(nitro) Isomers of Methyl Linolenate Contained in Isolated Fractions[a]

Fractions	Allylic nitrite(nitro) isomers
I	(9*c*,11*t*,15*c*-13AN)
II	(9*c*,13*t*,15*c*-12AN), (9*c*,13*t*,15*t*-12AN), and (9*c*,12*c*,14*t*-16AN)
III	(9*t*,11*t*,15*c*-13AN) and (9*c*,12*t*,14*t*-16AN)
IV	(10*t*,12*c*,15*c*-9AN) and (10*t*,12*t*,15*c*-9AN)

[a] A 25 × 0.46 cm silica column with hexane/isopropanol (99:1, vol/vol) at a flow rate of 1 mL/min was used for high-performance liquid chromatography (HPLC) analysis. The four peaks refer to the HPLC trace in Figure 4a.

Negative methane chemical ionization (NCI) was used to identify the presence of a nitrite and nitro group, to confirm the molecular weight and to locate the position of the functional group. The NCI spectra for the allylic nitrite(nitro) isomers of methyl linoleate (Table 5) and methyl linolenate (Table 9) display a *m/z* 46 and *m/z* 62 ion indicative of a nitro and nitrate functional group (20,22,23) and *m/z* 339 and *m/z* 337 molecular ions. These ions were not seen in the NCl spectra for the hydroperoxides of methyl linoleate (Table 5). The position of the functional group was determined by the aldehyde formed by thermal homolysis of the O-N bond of the nitrite group.

ACKNOWLEDGMENTS

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The Effect of Incubation Temperature on Yolk Lipid Parameters During Embryonic Development of the Alligator (*Alligator mississippiensis*)

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The effect of incubation temperature (30 and 33°C) on yolk lipid uptake and changes in the alligator has been studied. Notable changes occurred in the lipid and fatty acid compositions of the yolk. Triacylglycerols and phospholipids were the major lipid components of the yolk at the start of incubation. However, during incubation the level of cholesteryl esters increased considerably to become a major lipid component of the yolk at hatching. This increase in cholesteryl ester level occurred at a much earlier time in the eggs incubated at 33°C. The cholesteryl esters which accumulated within the yolk showed much higher levels of oleic acid. The triacylglycerols and phospholipids of the yolk both contained unusually high levels of palmitoleic acid. Their fatty acid compositions remained relatively unchanged during incubation. The lipid composition of the liver toward the end of the incubation period reflected the changes that occurred within the yolk. Thus the percentage of cholesteryl esters in the liver lipid of embryos incubated at 30 and 33°C were 7 and 40%, respectively. The accumulated cholesteryl esters also showed a significantly higher content of oleic acid. The differences in the yolk and tissue lipids during incubation at the two temperatures are discussed with reference to the respective rates of embryonic development but in particular with respect to the feature of temperature-dependent sex determination.

Lipids 28, 135–140 (1993).

Compared with the avian where the role of incubation temperature is almost wholly confined to being the final arbiter of survival, in the reptile the effects of incubation temperature are vividly manifested through a range of features. Thus in the crocodilians, the most extensively researched species to date, incubation temperature has been shown to control embryonic growth rate (1,2), the pigmentation pattern at hatching (3), post-hatching thermoregulation and growth (4,5) and, most importantly, the sex of the animal whereby at 30°C the hatchlings are all female, while at 33°C they are all male (1,6-9).

An extensive yolk mass to supply the developing embryo with a large proportion of its nutrients is common to all birds and reptiles (10–13). A major feature of the yolks is a high initial lipid content and rapid lipid utilization during the later stages of embryo development. Indeed, in the alligator there is a close similarity to the avian in the distinctive lipid compositional and metabolic events that accompany yolk uptake (11,12). The fact that temperature has an effect on overall yolk utilization has been shown in *Crocodylus johnstone* (10). Differences in lipid uptake at different incubation temperatures may be associated with or actually cause several of the events specified by incubation temperature in alligators. The present study reports on some of the major lipid changes occurring in the yolks of *Alligator mississippiensis* eggs during incubation at 30 and 33°C.

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Abbreviations: CE, cholesteryl esters; FFA, free fatty acids; FC, free cholesterol; PL, phospholipids; TG, triacylglycerols; UV, ultraviolet.

MATERIALS AND METHODS

Eggs of *Alligator mississippiensis* were collected from nests of wild alligators at the Rockefeller Wildlife Refuge, Grand Chenier, LA, on the first or second day after laying and were immediately transported by air to Manchester, England. On arrival at the laboratory (day 3/4 depending on the clutch), the eggs were weighed and placed in incubators accurate to ±0.01°C (Vinden Scientific Ltd., Bolton, England) maintained at 30 and 33°C. The relative humidity in the incubator was maintained as close as possible to 100% relative humidity.

Embryos and yolk complexes were carefully excised from four fertile eggs at days 8, 16, 24, 32, 40, 48, 56, 64 (hatching at 33°C) and 75 (hatching at 30°C) of incubation. The embryos were staged (Table 1) according to the method of Ferguson (8). Following weighing, the yolk complexes were individually homogenized and a known weight of the homogenate was removed. Total lipid from the homogenized sample was extracted by refluxing in a suitable excess of chloroform/methanol (2:1, vol/vol) according to well established procedures (14). Total lipid concentration was determined gravimetrically. Livers were excised from embryos at each incubation temperature just prior to hatching and the lipid extracted as for the yolk samples. All lipid extracts were then stored in chloroform at −20°C until analyzed.

The lipids were fractionated into their classes on thin-layer chromatoplates of silica gel G, thickness 0.25 mm, using a solvent system of hexane/diethyl ether/formic acid (80:20:1, vol/vol/vol). Following visualization and identification of the separated bands under ultraviolet (UV) light after spraying with a 0.1% (wt/vol) solution of 2,7-dichlorofluorescein in methanol, the phospholipids were recovered from the silica gel with 3 × 5 mL of chloroform/methanol/water (5:5:1, vol/vol/vol); the remaining

TABLE 1

Ages and Stages of Alligator Embryos from Which the Yolk Complexes Were Excised at 30 and 33°C Incubation Temperatures

Embryo age (d)	Stage	
	30°C	33°C
8	8	8
32	19	22
40	21	23
48	23	24
56	24	26
64	25	28 (hatching)
75	28 (hatching)	—

lipid fractions were recovered with 3 × 5 mL of diethyl ether. The lipid fractions were then transmethylated by refluxing with methanol/toluene/sulfuric acid (20:10:1, vol/vol/vol) in the presence of a pentadecanoic acid standard (15). Gas-liquid chromatography of the fatty acid methyl esters on a packed column of 15% CP Sil 84 on Chromosorb WHP (Chrompak UK Ltd., Middleburg, The Netherlands) enabled quantification of both the relative proportions of the major long-chain fatty acids present and the absolute amounts of the lipids. Quantification of the fatty acid chromatograms was by integration. Free cholesterol was quantified by charring and subsequent densitometry using a liquid scintillation counter (16). Identification of lipid and fatty acid fractions was by comparison with known standards. All solvents were distilled before use and where appropriate, operations were performed under an atmosphere of nitrogen.

RESULTS

The initial whole weights (±SE) of the eggs incubated at the two temperatures were: 30°C, 69.9 ± 1.11 g and 33°C, 69.8 ± 0.53 g. The weights of all the eggs thus fell within the range expected for those from mature alligators. Figure 1 shows the changes observed in the total yolk weights, total lipid content of the yolks and the percentage of lipids within the yolks during the incubations at 30 and 33°C. As can be seen, total yolk weights at both incubation temperatures remained very similar over the first 32 d of incubation, both groups registering very small decreases. Eggs incubated at 30°C continued to show only a further slight decrease in yolk weight up to 56 d of incubation, but thereafter there was a rapid decline. By contrast, eggs incubated at 33°C showed a rapid decrease in total yolk weight following 32 d of incubation (Fig. 1a). Just prior to hatching at 30 and 33°C, total yolk weights were 9.8 and 10.1%, respectively, of initial values. This overall difference was evident macroscopically, free yolk being present within the thick yolk sac membrane at 33°C but sparse at 30°C. The changes in total yolk weights during incubation at the two temperatures were mirrored by similar relative changes in total lipid contents. Although lipid absorption was particularly extensive during the last third of incubation, in the eggs incubated at 33°C mobilization was already prominent from a much earlier stage of incubation (Fig. 1b). By the end of incubation, some 85% of the initial yolk lipids had been absorbed in the eggs incubated at 30°C, but only some 73% in eggs incubated at 33°C. As incubation proceeded, total lipids assumed an increasing proportion of the total yolk weight in both groups of eggs, rising from an initial level of 20% of the yolk weight to 31% just prior to hatching (Fig. 1c).

Table 2 shows the changes that occurred in yolk lipid composition (major classes, weight percentage of total lipids) during incubation at 30 and 33°C. The reduction in lipid levels in the yolk was accompanied by substantial changes in composition, particularly in the eggs incubated at 33°C. Initially and throughout incubation in both groups of eggs, triacylglycerols were the principal lipid components accompanied also by a large proportion of phospholipids. Incubation was characterized by a large increase in the proportion of cholesteryl esters (CE) within the remnant yolk lipids of both groups of eggs; as yolk mobilization occurred, the proportion of CE increased

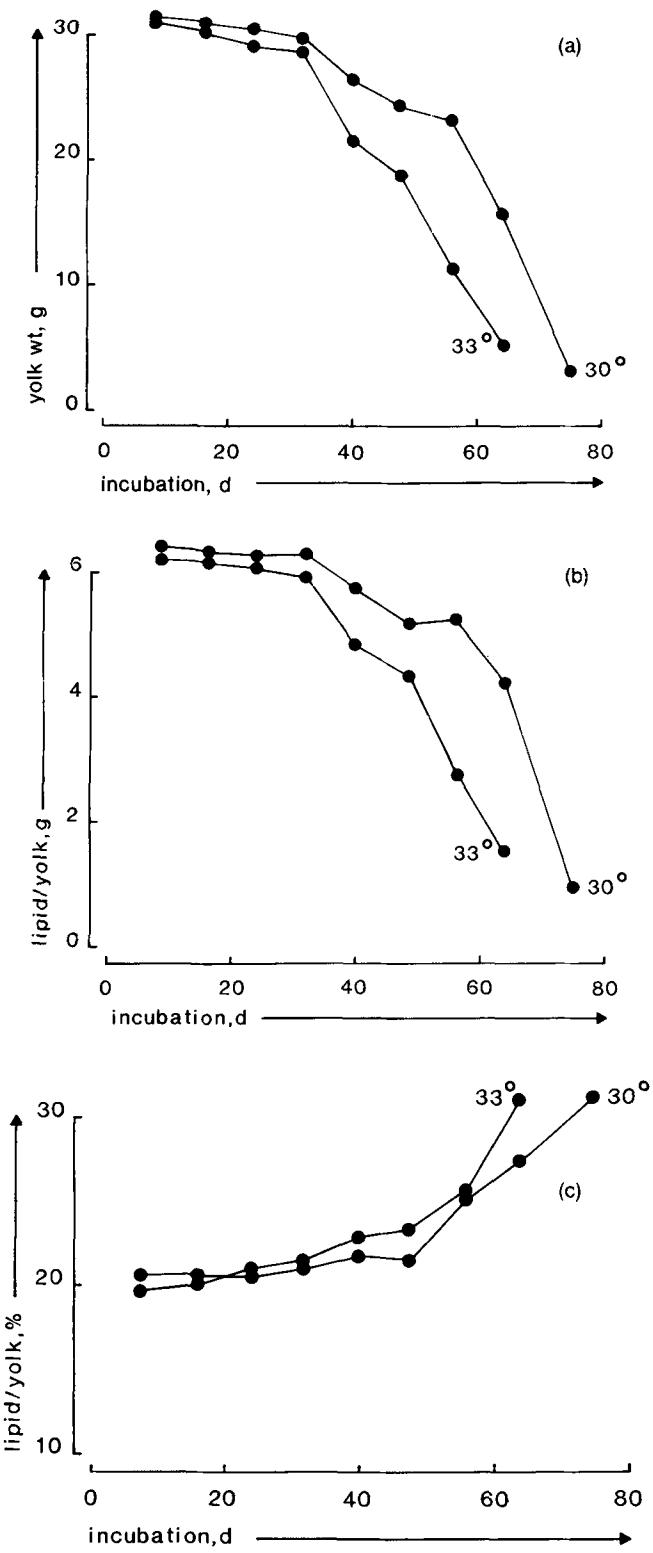

FIG. 1. Changes in total weight (a), total lipid content (b) and percentage of lipid (c) of the yolks during incubation at 30 and 33°C.

from a level of only about 1% in the yolk lipids initially to over 20% just prior to hatching. Proportions of free cholesterol were reduced. The increase in the level of CE was accompanied by a very large proportional decrease in phospholipids and also a decrease in triacylglycerols

TABLE 2

Lipid Composition (major fractions, percent of total lipid) of the Yolks During Incubation

Lipid composition[a]	Embryo age (d)							
	8	16	24	32	40	48	56	64	75
Incubation at 30°C									
CE	1.48 ± 0.06[b]	1.70 ± 0.34	1.27 ± 0.06	1.97 ± 0.16	2.39 ± 0.25	4.55 ± 0.19	3.76 ± 0.18	8.47 ± 0.32	20.8 ± 2.08
TG	69.5 ± 0.70	69.6 ± 1.12	69.7 ± 0.74	69.1 ± 0.07	68.9 ± 0.21	67.9 ± 1.12	72.5 ± 0.55	67.6 ± 0.56	59.4 ± 2.49
FFA	1.60 ± 0.08	1.66 ± 0.10	1.03 ± 0.06	1.53 ± 0.07	1.38 ± 0.03	2.15 ± 0.23	1.26 ± 0.04	2.81 ± 0.17	6.72 ± 0.39
FC	7.68 ± 0.21	6.62 ± 0.34	7.68 ± 0.47	6.04 ± 0.46	5.28 ± 0.48	5.89 ± 0.28	5.66 ± 0.47	5.32 ± 0.46	5.17 ± 0.60
PL	19.8 ± 0.74	20.5 ± 0.91	20.3 ± 1.21	21.3 ± 0.50	22.0 ± 0.56	19.5 ± 1.00	16.9 ± 0.44	15.8 ± 0.30	7.89 ± 0.49
Incubation at 33°C									
CE	1.23 ± 0.14	1.54 ± 0.15	1.72 ± 0.21	3.07 ± 0.10**[c]	3.49 ± 0.21*	5.39 ± 0.27*	8.43 ± 0.53***	20.4 ± 1.59***	
TG	66.4 ± 1.15	70.5 ± 0.49	70.0 ± 0.86	67.8 ± 1.03	66.0 ± 0.50**	67.6 ± 1.05	69.7 ± 0.33**	62.3 ± 1.71*	
FFA	1.40 ± 0.06	1.65 ± 0.07	1.07 ± 0.04	1.38 ± 0.06	1.87 ± 0.30	1.89 ± 0.20	2.46 ± 0.49*	3.05 ± 0.55	
FC	7.27 ± 0.34	5.63 ± 0.24	7.10 ± 0.85	6.16 ± 0.39	6.95 ± 1.43	7.73 ± 1.08	4.07 ± 0.35*	4.38 ± 1.05	
PL	23.7 ± 1.42	20.7 ± 0.28	20.1 ± 0.71	21.3 ± 0.82	21.7 ± 1.26	17.5 ± 0.81	15.4 ± 0.59	9.91 ± 1.23*	

[a]CE, cholesteryl esters; TG, triacylglycerols; FFA, free fatty acids; FC, free cholesterol; PL, phospholipids.

[b]Values are means ± SE.

[c]Significance of difference between values at 30 and 33°C: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(TG). At hatching the proportion of free fatty acids had shown an increase. Both groups of eggs showed similar yolk lipid compositions just prior to hatching. The changes in lipid composition of the yolk during incubation at 33°C were manifested at a very early stage; in particular the level of CE within the yolks had already shown a significant increase by day 32 of incubation. In general, if the data (Table 2) are compared by embryonic stage (Table 1), as opposed to embryonic age (days of incubation), then the changes in the lipid components are similar at equivalent embryonic stages regardless of incubation temperature, although some stage-independent changes exist.

Figure 2 shows the changes in the absolute amounts of CE, TG and phospholipids that occurred within the yolks during incubation at 30 and 33°C. As can be seen, in both groups of eggs a decrease in the amount of TG and phospholipids became increasingly obvious from around day 32 of incubation, but the reduction was particularly prominent in the yolks of the eggs incubated at 33°C. By contrast, absolute amounts of CE in the yolks of both groups of eggs increased over the incubation period with recognizable changes evident earlier in the eggs incubated at 33°C.

Table 3 shows the fatty acid compositions (major acids, weight percent of total) of the CE, TG and phospholipid fractions of the yolk at day 8 (beginning of incubation), day 64 (just prior to hatching at 33°C) and day 75 (just prior to hatching at 30°C) of incubation. In the triacylglycerol and phospholipid fractions, palmitic and oleic acids were the major constituent acids. Both fractions contained unusually high levels of palmitoleic acid. The high levels of polyunsaturated fatty acids in the phospholipids were characterized by levels of C_{20} and C_{22} acids that far exceeded those of the C_{18} precursors. In neither the TG nor the phospholipid fractions did the fatty acid compositions undergo any appreciable changes during the incubation period of the eggs. Although palmitic acid was the principal fatty acid present initially within the CE fraction, its level decreased markedly as incubation proceeded, and that of oleic acid increased. At day 64 of incubation there was a considerable difference in the oleic acid levels between the yolks of the two groups of eggs, but levels just prior to hatching were similar.

Table 4 shows the level and fatty acid composition of the CE found in the liver of the embryos at day 60 of incubation at 30 and 33°C. As can be seen, the concentration of CE within the lipids of the liver in embryos from the eggs incubated at 33°C was considerably elevated and showed a substantially different fatty acid composition, in particular much higher concentrations of palmitoleic, oleic and linolenic acids.

DISCUSSION

Yolk lipid uptake has been extensively studied in avian species, but particularly in the fowl (13,17). The patterns of events are similar throughout. A substantial reduction in egg yolk weight is associated with an extensive removal of lipids during the last third of the incubation period, the loss of lipids being particularly prominent in the last few days prior to hatching. Previous investigations (10,11) have already indicated that, in relative terms, yolk loss and lipid removal from the yolk of the alligator egg is

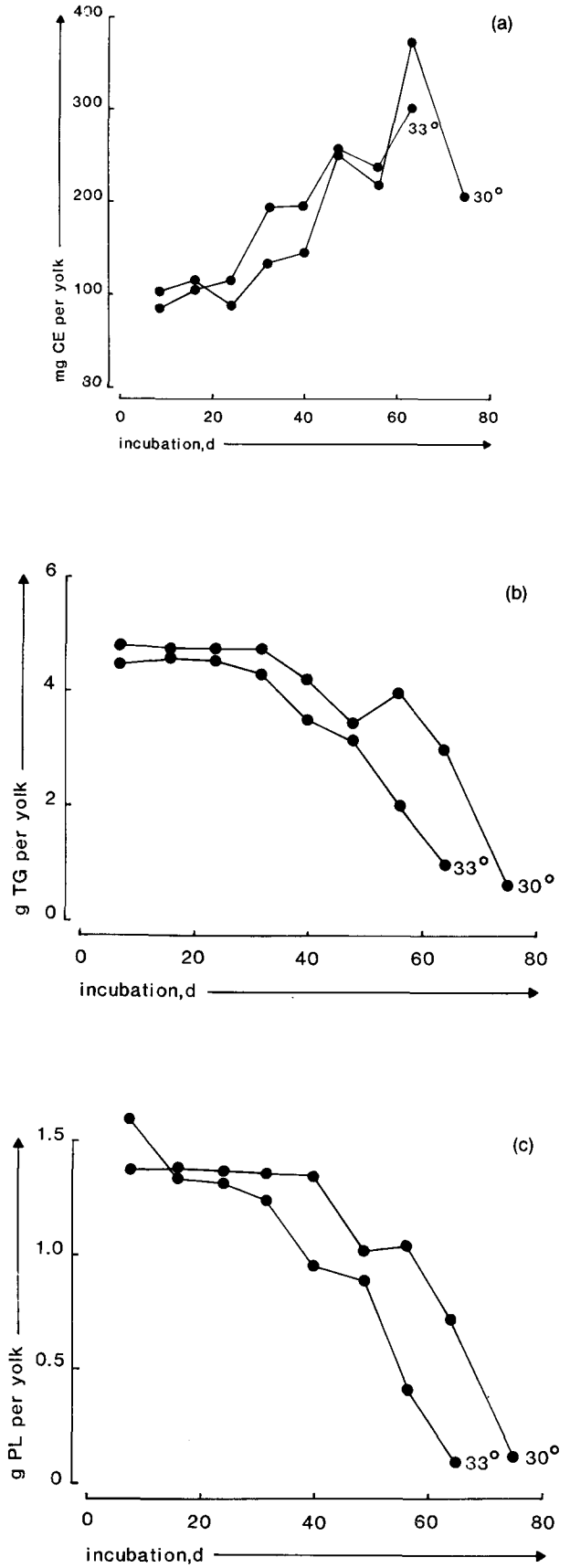

FIG. 2. Changes in the total weight of cholesteryl esters (CE) (a), triacylglycerols (TG) (b) and phospholipids (PL) (c) in the yolks during incubation at 30 and 33°C.

TABLE 3

Fatty Acid Compositions (major acids, percent of total) of the Cholesteryl Esters, Triacylglycerols and Phospholipids of the Yolks During Incubation as Function of Embryo Age (d) and Temperature[a]

	CE			TG			PL		
Fatty acids	8 d 30 + 33°C	64 d 33°C	75 d 30°C	8 d 30 + 33°C	64 d 33°C	75 d 30°C	8 d 30 + 33°C	64 d 33°C	75 d 30°C
Palmitic	60.0 ± 1.38	15.7 ± 1.34	14.0 ± 0.75	29.1 ± 0.15	23.6 ± 0.54	25.3 ± 0.41	32.1 ± 0.56	28.4 ± 0.37	34.4 ± 1.21
Palmitoleic	5.68 ± 1.70	8.01 ± 0.22	9.78 ± 0.53	18.5 ± 0.20	14.8 ± 0.50	15.8 ± 1.07	9.16 ± 0.27	8.80 ± 0.54	7.88 ± 0.37
Stearic	7.76 ± 0.60	4.32 ± 0.22	3.88 ± 0.15	6.49 ± 0.13	9.40 ± 0.24	9.27 ± 0.67	7.88 ± 0.37	7.75 ± 0.5	9.04 ± 0.57
Oleic	16.4 ± 0.83	55.7 ± 2.15	57.9 ± 1.14	32.3 ± 0.14	36.6 ± 0.51	35.0 ± 0.85	19.3 ± 0.37	22.3 ± 0.57	17.3 ± 0.92
Linoleic	4.47 ± 0.41	7.24 ± 0.20	5.72 ± 0.78	6.53 ± 0.12	9.59 ± 0.36	7.82 ± 0.57	4.18 ± 0.71	7.04 ± 0.61	4.29 ± 1.09
Linolenic	1.69 ± 0.25	4.11 ± 0.19	3.59 ± 0.74	4.58 ± 0.07	3.64 ± 0.21	3.24 ± 0.55	2.33 ± 0.37	3.00 ± 0.09	1.89 ± 0.45
Arachidonic	3.96 ± 0.61	2.29 ± 0.22	1.45 ± 0.15	1.02 ± 0.02	1.03 ± 0.12	1.09 ± 0.05	13.1 ± 0.26	11.5 ± 0.17	11.5 ± 0.34
Docosapentaenoic	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	2.38 ± 0.05	1.63 ± 0.04	1.70 ± 0.11
Docosahexaenoic	<1.0	1.56 ± 0.20	1.55 ± 0.13	<1.0	<1.0	1.73 ± 0.12	9.67 ± 0.21	9.28 ± 0.24	11.5 ± 0.57

[a]Values are means ± SE; abbreviations as for Table 2.

TABLE 4

Concentration and Fatty Acid Composition (major acids, percent of total) of the Cholesteryl Esters of the Liver at Day 60 of Incubation[a]

Lipid composition	Incubation temperature	
	30°C	33°C
% Cholesteryl esters in total lipid	7.47 ± 1.91[a]	39.5 ± 1.48***
Fatty acids		
Palmitic	42.1 ± 3.21	11.9 ± 0.55***
Palmitoleic	<1.0	6.91 ± 0.65***
Stearic	7.42 ± 0.41	2.37 ± 0.07***
Oleic	31.0 ± 2.84	48.6 ± 0.45**
Linoleic	8.55 ± 2.59	9.99 ± 0.89
Linolenic	<1.0	5.10 ± 0.13***
Arachidonic	5.53 ± 0.13	9.01 ± 0.23***
Docosapentaenoic	<1.0	1.18 ± 0.20
Docosahexaenoic	4.54 ± 2.62	5.80 ± 0.39

[a] Values are means ± SE. Significance of difference between values at 30 and 33°C: $^{**}P < 0.01$, $^{***}P < 0.001$.

similar to that displayed by the fowl egg. Increasing the temperature of incubation of the alligator egg in the present investigations clearly resulted in a significant effect on both the rate and extent of overall yolk weight reduction and lipid uptake by the embryo. Thus, although by the end of the incubation period at both temperatures the amount of yolk material remaining unabsorbed was only some 10% of that initially present in the egg, the amounts of yolk lipid remaining unabsorbed were 15 and 27%, respectively, at 30 and 33°C. A similar effect of increased incubation temperature on overall yolk lipid uptake has been observed in other reptilian species (10).

In general terms, the lipid composition of the yolk of the alligator egg is similar to that of the fowl (18). However, the extent of lipid compositional changes as incubation proceeds is far more pronounced in the alligator egg. Thus although there is an accumulation of CE within the yolk complex of the fowl during incubation, its extent is considerably less than for the alligator (4% compared to 20% of total lipids). Furthermore, absorption of yolk lipids by the fowl embryo does not display any overall preferential uptake of phospholipids; any preference is confined to specific species comprising the individual phospholipid fractions (13,19,20). The major influence on yolk lipid composition, especially during the latter part of incubation, is undoubtedly the increasing development of the yolk sac membrane (13,19). Its extensive function with respect to absorption and synthesis of lipids dictates the extent of lipid changes. Synthesis of CE, in particular cholesteryl oleate, by the yolk sac membrane in order to facilitate efficient transfer of lipid into the embryo has been shown to be a major function of the membrane's metabolism (21). Although it is tempting to suggest that the much higher accumulation of cholesteryl esters within the yolk of the alligator during incubation, compared to the fowl, may be associated with higher lipid concentrations within the egg when laid (11), the extent of the differences in the accumulations indicates a more fundamental metabolic reason, presumably differential synthesis by the yolk sac membrane.

It is clear from the present investigations that the incubation of eggs at 33°C considerably accelerated the processes of lipid removal and uptake by the embryo when compared to incubation at 30°C. Thus at 33°C, changes in the total yolk and lipid weights became particularly evident from as early as day 32 of incubation, that is halfway through the incubation period. By comparison, at 30°C large-scale changes in yolk uptake became evident from about day 50 of incubation, that is the last third of the incubation period (*cf.* the fowl). More definitive parameters confirm the earlier and more active lipid metabolism in the yolks at 33°C. Cholesteryl ester accumulation within the lipid of the yolk is the most prominent feature. In spite of the extensive mobilization of yolk lipids into the embryo in association with the cholesteryl esters synthesized (12), absolute cholesteryl ester levels within the yolk increased throughout the incubation period, whereas absolute levels of other major lipid components dramatically decreased. The earlier (in terms of age) changes in yolk lipid metabolism in embryos incubated at 33°C correlate with the faster general development and growth of such embryos (2).

As a result of yolk lipid mobilization, the tissues of the alligator embryo are undoubtedly exposed to increasingly high levels of cholesterol; the change in lipid composition of the liver is only the most obvious case of cholesterol accumulation arising from yolk lipid absorption (12,13). Much has been said of the unique feature of temperature-dependent sex determination displayed by the alligator (1,9). A whole range of possible determining factors involved in the process has been noted. Among the several hypotheses suggested to operate at a cellular level is the involvement of steroid hormone and accelerated gonadal differentiation during development at differing incubation temperatures (1). Differential testicular and ovarian steroidgenic enzyme mRNA in tissues during gestation has been identified in mammalian and avian species (22,23). It is therefore tempting to suggest that, arising from the temperature of incubation, the unique set of metabolic circumstances whereby the alligator embryo is exposed to widely differing levels of cholesterol during its development may promote the synthesis of particular patterns of steroid hormones from cholesterol with subsequent effects on sexual differentiation. Interestingly the time of primary temperature sex determination in the alligator extends to about stage 21 (day 40 at 30°C), the time when major differences in yolk lipids are evident. It seems likely that these lipid changes are involved in stimulation of the primary sexual phenotype and the development of secondary sexual states by affecting steroid hormone biosynthesis.

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METHOD

Determination of 4-Hydroxynonenal by High-Performance Liquid Chromatography with Electrochemical Detection

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4-Hydroxy-*trans*-2-nonenal (HNE) is a highly reactive product of lipid peroxidation originating from the breakdown of phospholipid-bound polyunsaturated fatty acids of cellular membranes. Despite its biological relevance, this aldehyde is only occasionally determined due to the complexity of previously described procedures. Here we present a simple and very sensitive method for the detection of HNE in biological samples. The method is based on the measurement of the 2,4-dinitrophenylhydrazone (DNPH) of the aldehyde by electrochemical detection after separation by reverse-phase high-performance liquid chromatography (HPLC). The greater sensitivity of this procedure as compared to the ultraviolet detection method commonly employed to measure DNPH derivatives of aldehydes after HPLC will allow the detection of HNE below the pmol level. The detection of HNE is highly reproducible even in normal tissues and cells. Increased amounts of HNE were detected in the livers of animals intoxicated with prooxidant agents such as carbon tetrachloride, bromotrichloromethane or bromobenzene. An exponential increase in HNE (and in malondialdehyde) was measured in peroxidizing liver microsomes (in the NADPH/Fe-dependent system). The method is also suitable for the study of very small samples, since HNE could be detected in approximately 1 million cultured cells (polyoma virus-transformed baby hamster kidney fibroblasts); the level rose after exposure of the cells to a Fe^{3+}/ADP prooxidant system.
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4-Hydroxy-*trans*-2-nonenal (HNE) is an α,β-unsaturated aldehyde which can be formed by peroxidation of ω6 unsaturated fatty acids, such as linoleic (18:2), γ-linolenic (18:3) and arachidonic (20:4) acid (1,2). In cellular membranes, however, HNE originates almost exclusively from phospholipid bound arachidonic acid (3) and can be considered as the most reliable index of free-radical stimulated lipid peroxidation. HNE exhibits a variety of cytopathological effects such as enzyme inactivation, inhibition of DNA and RNA synthesis, inhibition of protein synthesis and induction of heat shock protein (4-7). It is highly cytotoxic to many types of cells, such as hepatocytes, mammalian fibroblasts and Ehrlich ascites tumor cells (8-11); it shows inhibitory effects on cell proliferation as well as mutagenic effects (12,13). HNE may be one of the putative "second toxic messengers" (4,6) of peroxidation in cellular membranes, thereby providing the amplification that lipid peroxidation delivers for the initial free radical attack.

A number of methods have been applied to detect HNE in biological systems. The first to be developed (1,14) employed the commonly used aldehyde-derivatizing agent, 2,4-dinitrophenylhydrazine (DNPH) to stabilize the otherwise volatile HNE and to facilitate its detection both in a preliminary purification stage using thin-layer chromatography (TLC) and during its separation using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 378 nm (molar absorptivity of the DNPH derivative = 25000-28000, λ = 360-380 nm). A method for the HPLC determination of free HNE has also been described (15). The detection limit of standard HNE is approximately 2 pmol and that in biological samples is approximately 10 pmol/mg protein. Another HPLC method (16) employs derivatization with a fluorescent reagent (1,3-cyclohexanedione), but in our experience, the lack of a purification stage renders the HNE peak hardly distinguishable in chromatograms of biological samples. A very recent method (17) employing TLC densitometry to quantify aldehydes using the two derivatizing agents described above is limited by its low sensitivity. Various determinations of free HNE using gas chromatography/mass spectrometry (GC/MS) have also been reported (18-20). These are extremely sensitive and specific, but place a heavy burden on resources.

In order to overcome these problems of sensitivity or cost, we have modified the early DNPH derivatization method and employed electrochemical detection to improve the detection limit. This procedure has enabled us to measure HNE in very small biological samples, in both *in vivo* and *in vitro* experiments.

MATERIALS AND METHODS

Chemicals. Chemically synthesized 4-hydroxynonenal was a gift from H. Esterbauer, Department of Biochemistry, University of Graz, Graz, Austria. 2,4-Dinitrophenylhydrazine (analytical grade) was from Merck (Darmstadt, Germany). Dichloromethane, chloroform, benzene, *n*-hexane and methanol (all HPLC grade) were from Baker (Phillipsburg, NJ).

DNPH derivatization. DNPH derivatization of HNE essentially followed the procedure of Benedetti *et al.* (21). Samples obtained from *in vivo* and *in vitro* experiments were immediately reacted with an equal volume of DNPH reagent (pellets of approximately 10 million cells were derivatized with 2 mL of reagent). The reaction was carried out in the dark for 2 h at room temperature. The DNPH reagent was freshly prepared by dissolving 50 mg of DNPH in 100 mL of 1 N HCl, and the reagent was extracted twice with 50 mL of hexane to remove impurities. The reaction mixture (sample plus DNPH reagent) was

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; DNPH, 2,4-dinitrophenylhydrazine; ED, electrochemical detection; EDTA, ethylenediaminetetraacetic acid; GC/MS, gas chromatography/mass spectrometry; HNE, 4-hydroxy-*trans*-2-nonenal; HNE-DNPH, 2,4-dinitrophenylhydrazone of 4-hydroxynonenal; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PBS, phosphate buffered saline; TBA, 2-thiobarbituric acid; TLC, thin-layer chromatography; UV, ultraviolet.

extracted three times with 3 vol of dichloromethane. The pooled extracts were dried under N_2 and redissolved in 200 μL of chloroform. The sample was then subjected to a purification procedure using either TLC or solid phase extraction. The DNPH derivatives (including the HNE-DNPH) were recognizable during these procedures because of their intense yellow color.

TLC purification. The sample was applied to a TLC plate (20 cm × 20 cm × 0.2 mm, silica gel pre-coated, obtained from Merck) and developed twice with dichloromethane (5 cm) and once with benzene (9 cm) (21). Standard HNE-DNPH was run along either end of the plate. The area corresponding to standard HNE-DNPH, together with material migrating at a higher R_f up to unreacted DNPH, was scraped off and eluted three times with 2 mL of methanol. The methanol extracts were pooled, dried under N_2 and redissolved in methanol (200 μL) prior to HPLC analysis.

Purification by solid phase extraction. The sample was applied to a disposable silica gel extraction column (3 mL vol, Baker) which had been preequilibrated with *n*-hexane/chloroform (2:1, vol/vol). The same solvent mixture (approximately 10 mL) was used to wash off the highly lipophilic DNPH derivatives in the sample in a discrete band. The remaining material (including the HNE-DNPH) was eluted using chloroform (approximately 6 mL). The chloroform extract was evaporated under N_2 and redissolved in methanol (200 μL) prior to HPLC analysis.

HPLC analysis. The separation of the HNE-DNPH derivative was performed using reverse-phase HPLC. A C18 column (5 nm Ultrasphere; 25 cm × 4.6 mm i.d.; Beckman Instruments Inc., San Ramon, CA), protected by a C18 precolumn (Guard-Pak, Millipore, Milano, Italy), was used. The mobile phase, a mixture of 30 mM sodium citrate/27.7 mM acetate buffer, pH 4.75, with three volumes of methanol, was delivered isocratically at 1.0 mL/min, using a Beckman (Palo Alto, CA) HPLC pump, model 114 M. Dinitrophenylhydrazones, including HNE-DNPH, were detected using an ESA model 5100A Coulochem detector linked to an ESA model 5011 analytical cell (ESA Inc., Bedford, MA). A potential of +0.3 V was used at the upstream (screening) electrode; the downstream (detecting) electrode was set at +0.8 V. These potentials were found in preliminary experiments as the most suitable conditions for maximal detector yield of HNE-DNPH together with maximal screening. Standards and samples were injected using a Rheodyne syringe loading injector model 7125 (Rheodyne, Cotati, CA) fitted with a 6-μL loop. The identification of HNE-DNPH was made on the basis of the retention time of standard HNE-DNPH, as well as on the basis of the coelution test performed by adding HNE-DNPH to biological samples. Quantitation of the HNE-DNPH derivative in standards and samples was done by measurement of peak height.

In vivo *experiments.* Male NMRI albino mice (Charles River, Como, Italy) weighing 20–30 g were used. The animals were maintained on a complete pellet diet (Altromin-Rieper, Bolzano, Italy). They were starved overnight before intoxication. CCl_4, $BrCCl_3$ or bromobenzene was administered intragastrically under light anesthesia, at the dose of 26, 26 and 15 mmol/kg body wt, respectively. Bromobenzene had been pre-mixed with two volumes of mineral oil. Control animals were given an equal volume of mineral oil. The animals were sacrificed 2, 3 and 12 h after intoxication, respectively. Liver homogenates (20%) were prepared in 0.154 M KCl/3 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4), and aliquots were taken for DNPH-derivatization, estimation of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA; ref. 22), and protein determination (23).

In vitro *experiments.* Rat liver microsomes were prepared and allowed to peroxidize in a NADPH/Fe-dependent system as described by Benedetti *et al.* (24). Two concentrations (6 and 60 μM) of $FeSO_4$ were used to promote lipid peroxidation. At the times indicated, 1.5 mL of the mixture was removed for DNPH derivatization, and 1.0 mL was removed for MDA estimation. An aliquot was also removed for protein determination.

Baby hamster kidney fibroblasts transformed using polyoma virus (BHK-21/Py Y) (25) were routinely grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco Products Ltd., Uxbridge, United Kingdom), containing 10% (vol/vol) fetal calf serum (Flow Laboratories Ltd., Irvine, United Kingdom), under an atmosphere of air containing 5% CO_2. No evidence of mycoplasmal contamination was found by staining with bisbenzamide (Hoechst 33258, Sigma, Poole, United Kingdom). The cells were grown to near confluency in 80-cm^2 culture flasks (Nunc, Roskilde, Denmark) and washed twice with DMEM. Half were subjected to prooxidant stress, induced by a Fe^{3+}/ADP-containing medium (374 μM $FeCl_3$/10 mM ADP dissolved in DMEM) for two hours. DMEM alone was used as the medium for the remaining control monolayers. The prooxidant and the control media were then removed from the monolayers, which were washed twice with DMEM, trypsinized, washed once in phosphate buffered saline (PBS), suspended in PBS and counted using a Coulter counter (Coulter Electronics Ltd., Luton, United Kingdom). The cell suspension was then centrifuged, and the cell pellet was derivatized using the DNPH reagent.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of standard HNE-DNPH revealed by electrochemical detection (ED). The detection limit for standard HNE-DNPH was 15 fmol (with a signal-to-noise ratio of 4). Thus the sensitivity of this method seems to be far greater than that of other methods (with the exception of GC/MS) employed to measure HNE. We found good proportionality between the amount of HNE-DNPH injected onto the column and the detector response. A typical calibration curve is shown in Figure 2.

We then applied this HPLC-ED method to biological samples obtained from *in vivo* and *in vitro* systems. The original method of Benedetti *et al.* (1) describes a sample purification using TLC. Because of the notoriously low recovery of analytes separated by TLC, we initially developed an alternative purification procedure for our *in vivo* samples, which utilized solid phase extraction with silica gel columns. This procedure was also considered to be more convenient and less time-consuming than TLC. Therefore, it was used for the determination of HNE in liver samples obtained from CCl_4, $BrCCl_3$ or bromobenzene intoxicated mice. Figure 3 shows representative chromatograms for control and CCl_4 intoxicated hepatic samples. As can be seen in Table 1, the highest levels of HNE were found in bromobenzene hepatotoxicity (in

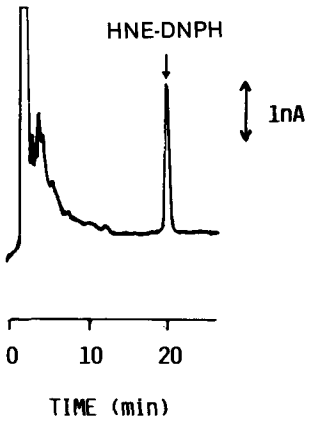

FIG. 1. High-performance liquid chromatography elution profile of standard 2,4-dinitrophenylhydrazone of 4-hydroxynonenal (HNE-DNPH) (0.5 pmol) with electrochemical detection. A C18 reverse phase column was used. Mobile phase, methanol/30 mM sodium citrate/27.7 mM acetate buffer, pH 4.75 (75:25, vol/vol) delivered isocratically at 1.0 mL/min. The HNE-DNPH was dissolved in methanol. A screening potential of +0.3 V and a detecting potential of +0.8 V were used.

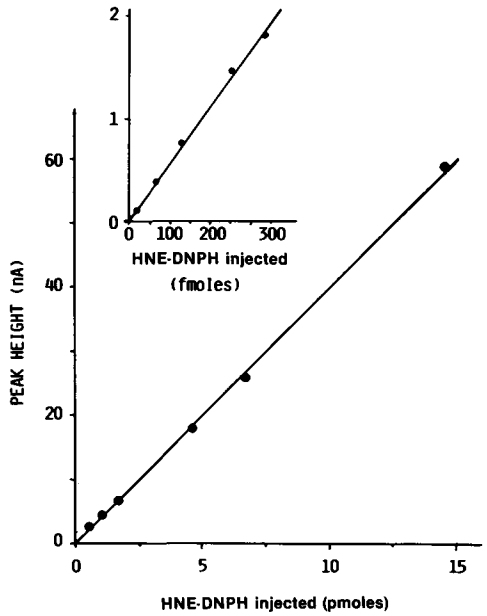

FIG. 2. Calibration curve of standard 2,4-dinitrophenylhydrazone of 4-hydroxynonenal (HNE-DNPH) by high-performance liquid chromatography (HPLC) with electrochemical detection. HPLC analysis was carried out as described in Figure 1.

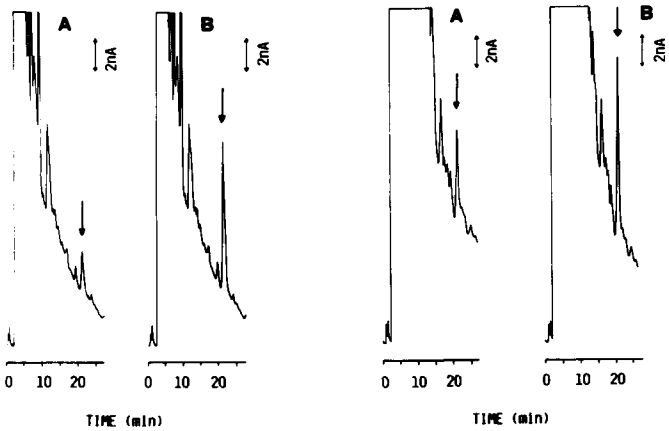

FIG. 3. Left: High-performance liquid chromatography (HPLC) analysis with electrochemical detection of (A) a liver extract from a control mouse (3.4 pmol 4-hydroxy-*trans*-2-nonenal (HNE)/mg protein), and (B) the same extract to which standard 2,4-dinitrophenylhydrazone (HNE-DNPH) had been added. Right: HPLC analysis with electrochemical detection of (A) a liver extract from a CCl_4 intoxicated mouse (7.8 pmol HNE/mg protein), and (B) the same extract to which standard HNE-DNPH was added. The HNE-DNPH peaks are indicated by arrows. Samples derived from a portion of liver corresponding to approximately 0.2 mg of protein were injected.

TABLE 1

Hepatic Concentrations of HNE and MDA in Control and CCl_4, $BrCCl_3$ or Bromobenzene Intoxicated Mice[a]

Treatment	(n)	HNE (pmol/mg protein)	MDA (pmol/mg protein)
Control	6	4.5 ± 0.7	18 ± 3
CCl_4	3	11.3 ± 0.7	33 ± 7
$BrCCl_3$	3	26.2 ± 3.8	40 ± 4
Bromobenzene	3	90.6 ± 19.3	1251 ± 420

[a]HNE was determined by high-performance liquid chromatography with electrochemical detection. MDA was determined with thiobarbituric acid. The animals were intoxicated and sacrificed at different times as described in Materials and Methods. Results are means ± SE. Abbreviations: HNE, 4-hydroxy-*trans*-2-nonenal; MDA, malondialdehyde.

agreement with our previous results; ref. 21) and the lowest in CCl_4 and $BrCCl_3$ hepatotoxicity. The basal levels of HNE measured by this method in normal livers are quite similar to that reported by others (20,26) and a little lower than that reported by Yoshino *et al.* (27). Data on the hepatic content of MDA are also reported here for comparison. Although the production of HNE is lower as compared to that of MDA, it must be considered, as is known, that HNE is a much more specific product of lipid peroxidation.

As stated in the Materials and Methods, the identification of the HNE-DNPH peak was done on the basis of the retention time of the standard (Fig. 4) and on the basis of the coelution test (Fig. 3). Further evidence for the identification of HNE-DNPH was obtained by comparing the modifications of the peak of standard HNE-DNPH (Table 2) and of putative HNE-DNPH in biological samples (Table 3), following changes in oxidative potential. Tables 2 and 3 show that the changes in the two peaks were very similar when the oxidative potential was varied.

Figure 5 shows the results of an *in vitro* experiment in which liver microsomes were allowed to peroxidize in a NADPH/Fe^{2+}-dependent system (with 6 or 60 μM $FeSO_4$). In both cases an exponential increase of HNE was observed during the incubation, and this was paralleled by an increase in MDA. The molar ratio of HNE/MDA was 1:5–10 in the system in which lipid peroxidation was induced by the lower amount of iron (6 μM), and 1:20–40 in the system in which lipid peroxidation was

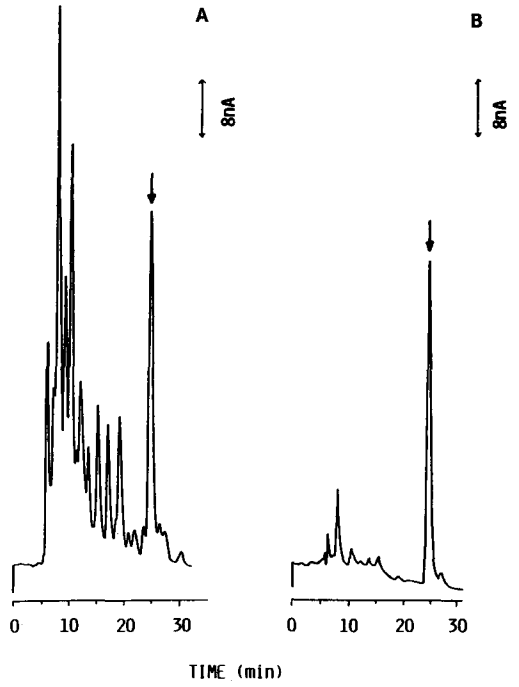

FIG. 4. High-performance liquid chromatography analysis with electrochemical detection of (A) a liver extract from a bromobenzene intoxicated mouse (66 pmol 4-hydroxy-*trans*-2-nonenal/mg protein). A sample derived from a portion of liver corresponding to approximately 0.2 mg of protein was injected. Standard 2,4-dinitrophenylhydrazone (HNE-DNPH) is shown in (B) for comparison of retention time. The HNE-DNPH peaks are indicated by arrows.

TABLE 2

Changes in the Response of the Electrochemical Detector with Changes in the Oxidative Potential Applied to Standard HNE-DNPH[a]

Detector potential	Detector response (nA) on injection of 20.6 pmol HNE	% of response at +0.8 V
+0.80	80	100
+0.75	73	91
+0.70	55	69

[a]Abbreviations: HNE-DNPH, 2,4-dinitrophenylhydrazone; HNE, 4-hydroxy-*trans*-2-nonenal.

induced by the higher amount of iron (60 μM). Thus the ratio of HNE/MDA was higher when there was a lower degree of lipid peroxidation. The reason for such a difference is presently under investigation. Nevertheless, in the *in vivo* experiments too, a similar change in the ratio HNE/MDA was observed with different extents of lipid peroxidation (Table 1).

When very low levels of HNE were to be measured, in some samples the solid phase extraction for the purification step was found to be insufficient to remove material that interfered with the detection. Therefore it was considered necessary to revert to the TLC method to purify such samples. Using the procedure described in Materials and Methods (*i.e.*, scraping a broader area than that strictly corresponding to standard HNE-DNPH), we have obtained recoveries of HNE-DNPH added to rat liver

TABLE 3

Changes in the Response of the Electrochemical Detector with Changes in the Oxidative Potential Applied to a Sample Derived from the Liver of a Bromobenzene-Intoxicated Mouse

Detector potential	Detector response (nA)	% of response at +0.8 V
+0.80	82	100
+0.75	75	92
+0.70	52	63

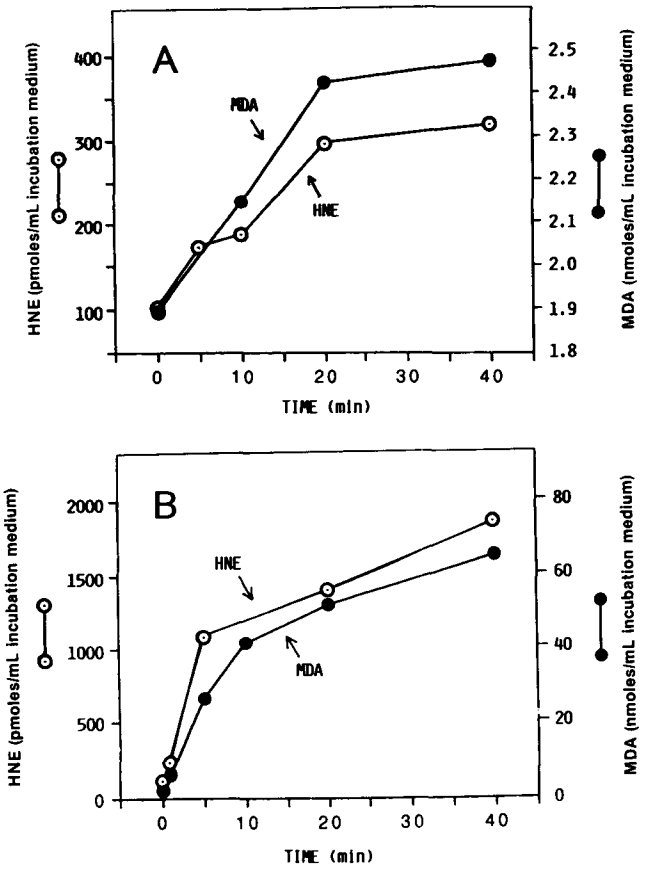

FIG. 5. Time-course of formation of 4-hydroxy-*trans*-2-nonenal (HNE) and concomitant evolution of malondialdehyde in liver microsomes peroxidized in the NADPH/Fe-dependent system. The incubation mixture was composed of microsomes derived from 250 mg liver (or 5 mg protein)/mL, 0.15 M KCl, 0.05 M tris-maleate buffer, pH 7.4, 6 or 60 μM $FeSO_4$, and an NADPH-generating system (0.1 mM NADP, 5 mM $MgCl_2$, 2.5 mM nicotinamide, 3 mM DL-isocitrate and 0.1 μM units/mL isocitrate dehydrogenase) (Boehringer Mannheim, Milano, Italy). (A), 6 μM $FeSO_4$; (B) 60 μM $FeSO_4$. MDA, malondialdehyde.

homogenates of 90 ± 8.5% (n = 6). This compares favorably with the usual virtually complete recoveries from solid phase extraction. The recovery of non-derivatized HNE added to the buffer alone (0.154 M KCl/3 mM EDTA, pH 7.4) and processed the same as liver homogenates or microsomal suspensions was 87 ± 2.8% (n = 4) when TLC purification was used, and almost complete (97%, mean of two samples) when the solid phase extraction was utilized. When HNE was added to liver

TABLE 4

Levels of HNE in Cultured BHK-21/PyY Cells Subjected to Oxidative Stress Induced by Fe^{3+}/ADP[a]

Treatment	HNE (pmol/million cells)
Control	1.96
$+Fe^{3+}$/ADP	7.84

[a]BHK-21/PyY cells were grown to near confluency in DMEM containing 10% fetal calf serum washed with DMEM, and then subjected to oxidative stress by adding DMEM containing 374 μM $FeCl_3$ and 10 mM ADP. DMEM alone was added to control cultures. Abbreviations: HNE, 4-hydroxy-*trans*-2-nonenal; DMEM, Dulbecco's Modified Eagle's Medium.

homogenates before the derivatization step, the recovery was much lower (nearly 40%), whatever purification procedure was used. This was very likely due to the quick binding of the aldehyde to biological components, as has also been noted by others (26).

TLC was used to purify samples obtained from cell culture experiments. The results of such measurements on control and oxidatively stressed fibroblasts are shown in Table 4. This indicates the suitability of the HPLC-ED method for the investigation of HNE in small *in vitro* samples such as are commonly obtained in cell culture work.

In summary, we have developed a sensitive method for the analysis of HNE in biological samples. The lowest amount of HNE which can be measured is 0.5–1 pmol per injected sample. In the *in vivo* studies, the injected samples were derived from a portion of liver corresponding to approximately 0.2 mg protein. Thus tissue samples much smaller than those used in our previous study (liver corresponding to 5 mg protein; ref. 21) can be analyzed. The method appears particularly suited, for example, to the measurement of endogenous levels of HNE under circumstances in which low levels of lipid peroxidation have occurred, or when the sample size is small (*e.g.*, in microassays, cell culture work or biopsy samples).

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Regiospecific Analysis of Triacylglycerols Using Allyl Magnesium Bromide

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A method for the regiospecific analysis of triacylglycerols (TAG), using the Grignard reagent allyl magnesium bromide (AMB) to partially deacylate TAG, is described. 1,3-Distearoyl-2-oleoyl-glycerol (SOS) and 1,3-didecanoyl-2-palmitoyl-glycerol (CPC) were reacted with AMB. From the resulting mixture, the four different classes of partial acylglycerols and TAG were isolated, and the mole ratios between stearic acid and oleic acid, or decanoic acid and palmitic acid, respectively, were determined in each fraction. Different approaches of calculating the composition of the fatty acids in positions *sn*-1(3) and *sn*-2 of the original TAG were compared. For the *sn*-2 position, the best estimate was the direct determination of the fatty acid composition of 2-monoacylglycerol (MAG). Mole percentages of stearic acid and decanoic acid in the *sn*-1(*sn*-3) positions of SOS and CPC, respectively, were most accurately estimated from the fatty acid compositions of TAG and 2-MAG according to the formula: $1.5 \times \text{TAG} - 0.5 \times 2\text{-MAG}$. Using AMB and the present method of calculation, the results obtained were more accurate and showed smaller standard deviations than those obtained using other common deacylating agents, such as ethyl magnesium bromide or pancreatic lipase.
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The distribution of fatty acids between the primary and secondary positions in triacylglycerols (TAG) can be determined either after pancreatic lipase degradation or after chemical degradation using a Grignard reagent (1,2). Both methods are based on the assumption that partial deacylation of TAG leads to representative mixtures of diacylglycerols (DAG) and monoacylglycerols (MAG), which can be characterized by chromatographic analysis. These methods, however, have certain limitations. The lipase assay is not reliable for TAG which contain significant amounts of short- or medium-chain fatty acids (C_{10} or less) or very long-chain polyunsaturated fatty acids (PUFA) (1). Milk fat, for instance, will not give representative acylglycerols as short- and medium-chain fatty acids are more readily hydrolyzed by the pancreatic lipase than are long-chain fatty acids (2). Some PUFA, on the other hand, have been reported to resist hydrolysis by lipase (3-5); therefore, marine oils should not be analyzed by the lipase assay either.

The reaction of TAG with ethyl magnesium bromide (EMB) can be used to obtain representative mixtures of DAG (6). However, the MAG are easily isomerized and cannot be used for regiospecific analysis of TAG. Therefore the mole percentages of the fatty acids in the *sn*-1(*sn*-3) and *sn*-2 positions are calculated from the composition of TAG and 1,2(2,3)-DAG. In the present communication it is shown that the more reactive Grignard reagent allyl magnesium bromide (AMB) yields representative 2-MAG, allowing the composition of the native TAG *sn*-2 position to be determined directly. The composition in the *sn*-1(*sn*-3) position can then be estimated from the composition of 2-MAG and TAG. The estimates obtained using AMB compare favorably to those found using EMB as they come closer to the theoretical value and have smaller standard deviations.

MATERIALS AND METHODS

1,3-Distearoyl-2-oleoyl-*sn*-glycerol (SOS) (99%+) was purchased from Sigma Chemical Company (St. Louis, MO). 1,3-Didecanoyl-2-palmitoyl-*sn*-glycerol (CPC) was synthesized according to Redgrave *et al.* (7). The structure of 1,3-didecanoyl-*sn*-glycerol was verified by ^{1}H nuclear magnetic resonance. Silicic acid 60G thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) impregnated with boric acid were used for separation of the different acylglycerol species resulting from the action of AMB on TAG. The plates were prepared by spraying with a 0.4 M aqueous boric acid solution until saturated. Then, the plates were dried at room temperature overnight and kept in a desiccator over a drying agent. Diethyl ether, analytical grade, was redistilled immediately before use. During distillation, the ether was protected from the moisture by a $CaCl_2$-tube. AMB in diethyl ether (2M) was synthesized according to Gilman and McGlumphy (8). Methanol and hexane were high-performance liquid chromatography-grade and obtained from Rathburn Chemicals (Walkerburn, Scotland). Anhydrous Na_2SO_4, 37% HCl (aq.), and boric acid were purchased from Merck. Na_2SO_4 was dried at 130°C for at least 4 h before use. Dichlorofluorescein and tripalmitoylglycerol were from Sigma.

The TAG to be analyzed (6.0 to 6.5 mg) was dissolved in diethyl ether (5 mL) in a Teflon capped reaction tube with magnetic stirring. AMB (200 μL) was added with a pipette that had been flushed with nitrogen; the diethyl ether solution became opaque, indicating a spontaneous reaction. After one minute, additional diethyl ether (5.0 mL) was added. Then the organic phase was washed, first with an acidic buffer (4.0 mL) prepared by adding 37% HCl (1.0 vol) to a 0.4 M boric acid solution (36 vol) giving a final concentration of 0.27 M. This neutralized the $Mg(OH)_2$ formed in the reaction mixture upon addition of water. Additional washings (2×4.0 mL) with a 0.4 M boric acid solution removed excess HCl and remaining magnesium salts. The ether phase was dried briefly with about 2 g of anhydrous Na_2SO_4 before being decanted and evaporated *in vacuo*. The acylglycerol mixture was redissolved in chloroform (400 μL).

The chloroform solution was applied to a boric acid impregnated TLC plate which was developed in chloroform/acetone (96:4, vol/vol) in a saturated chamber. The fractions were visualized by spraying with 2,7-dichlorofluorescein (0.1% wt/vol) in anhydrous ethanol. The following bands were observed: 1,3-MAG ($R_f = 0.12$); 2-MAG ($R_f = 0.20$); 1,2(2,3)-DAG ($R_f = 0.50$); 1,3-DAG ($R_f = 0.62$);

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Abbreviations: AMB, allyl magnesium bromide; CPC, 1,3-didecanoyl-2-palmitoyl-*sn*-glycerol (10:0/16:0/10:0); DAG, diacylglycerol; EMB, ethyl magnesium bromide; MAG, monoacylglycerol; PUFA, polyunsaturated fatty acid; SOS, 1,3-distearoyl-2-oleoyl-*sn*-glycerol (18:0/18:1/18:0); TA, tertiary alcohols; TAG, triacylglycerol, TLC, thin-layer chromatography.

tertiary alcohols of the deacylated fatty acids (TA) (R_f = 0.70); and TAG (R_f = 0.82). All bands except TA were scraped off. The acylglycerols were extracted from the silicic acid with diethyl ether (2 × 2 mL). After brief centrifugation, the ether was decanted and evaporated under a stream of N_2. The residue was methylated using the alkaline transesterification procedure described by Christie (9). The methyl esters from SOS were analyzed on a Hewlett-Packard (Palo Alto, CA) Gas Chromatograph HP 5890A, equipped with a flame-ionization detector, using a HP 7673A Automatic Sampler. The fused silica column (30 m in length and 0.32 mm i.d.) coated with SP2330 (film thickness 0.25 μm) was from Supelco (Bellefonte, PA). A helium flow rate of 34 mL/min and a split ratio of 1:20 were used. The temperature of the oven was initially 140°C and was increased at a rate of 3.0°C/min until the final temperature (200°C) was reached. The methyl esters from CPC were analyzed on a Hewlett-Packard Gas Chromatograph HP 5830A equipped with a flame-ionization detector and a column (2.75 m × 2 mm) containing 10% SP 2330 on 100/120 Chromosorb WAW (on column injection) using an HP 7671A automatic sampler. A flow rate of 40 mL helium/min was employed. After the injection of a sample, the column was maintained at 100°C for 4 min before the temperature was increased to 220°C at a rate of 8°C/min. In both gas chromatographs the detector and the injector were maintained at 250°C.

The composition of the partial acylglycerols was calculated based on relative integrated peak area obtained by gas chromatography for the pure triacylglycerols.

RESULTS AND DISCUSSION

The results of the analyses of the partial acylglycerols obtained after AMB degradation of SOS and CPC, respectively, are shown in Table 1.

For SOS, the stearic acid accounted for more than 98% of the total fatty acids in the 1,3-DAG and only 2.4% of the total fatty acids in the 2-MAG, showing that a representative mixture of partial acylglycerols was obtained. In the 1(3)-MAG from SOS, oleic acid originating from isomerized 2-MAG comprised 6.8% of the fatty acids. The presence of stearic acid (54%) in 1,2(2,3)-DAG may correspondingly be explained by minor isomerization of 1,3-DAG. The remaining TAG are still representative as shown by the presence of 66.7% stearic acid.

For CPC, the 2-MAG was only contaminated with decanoic acid to an extent of 1.2%. The 1(3)-MAG, on the other hand, contained 7.7% palmitic acid which had migrated from the 2-position. As found for SOS, the diglycerides of CPC were slightly less pure.

For other TAG species, such as 1,3-dipalmitoyl-2-oleoyl-glycerol and 1,3-didecanoyl-2-stearoyl-glycerol, comparably low degrees of migration for 2-MAG and 1,2(2,3)-DAG were observed (C.C. Becker, unpublished data).

The objective of a regiospecific analysis is to determine the fatty acid composition of positions *sn*-1(3) and *sn*-2 in the original TAG. This can be done by direct determination or by calculation based on the composition of the different acylglycerols, as demonstrated in Tables 2 and 3.

For both SOS and CPC the most accurate estimate for the composition of 1(3)-MAG was obtained by the formula: 1.5 × TAG − 0.5 × 2-MAG. The estimates were 98.8 ± 0.7 percent stearic acid in SOS and 99.4 ± 1.4 percent decanoic acid in CPC. For the 2-MAG obtained from the deacylation of TAG, only a low level of migration had occurred, and the composition of the 2-position could therefore be determined directly. During the synthesis of the TAG used in this investigation, a limited isomerization of the intermediate 1,3-DAG may have taken place. The values calculated for the *sn*-1(3) positions are therefore probably even less than one percent from the true value. For both 1(3)-MAG and 2-MAG it should be noted that the procedures giving the best estimates of the fatty acid composition in 1(3)-MAG and 2-MAG are also the procedures with the lowest standard deviation.

It is surprising that while the 1(3)-MAG resulting from the Grignard deacylation is slightly contaminated with former 2-MAG, the 2-MAG obtained appears to be almost completely representative. This could be explained by the greater stability of 1(3)-monoacylglycerols; thus an equilibrium mixture of monoolein in an aqueous system consists of about 90% of the 1-isomer and 10% of the 2-isomer (10).

When the EMB method is used, the composition of

TABLE 1

Fatty Acid Composition (mole% decanoic acid or stearic acid) of the Acylglycerols from 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) After Deacylation by the Grignard Reagent Allyl Magnesium Bromide[a]

TAG analyzed	Acylglycerol formed	%18:0 (for SOS)[b] %10:0 (for CPC)[b]	%18:1 (for SOS)[c] %16:0 (for CPC)[c]
SOS	1(3)-MAG	93.2 ± 3.8	6.8
	2-MAG	2.4 ± 0.8	97.6
	1,2(2,3)-DAG	53.9 ± 3.6	46.1
	1,3-DAG	98.3 ± 1.7	1.7
	TAG	66.7 ± 0.4	33.3
CPC	1(3)-MAG	92.3 ± 1.4	7.7
	2-MAG	1.2 ± 1.3	98.8
	1,2(2,3)-DAG	51.7 ± 1.0	48.3
	1,3-DAG	93.8 ± 2.1	6.2
	TAG	66.7 ± 0.8	33.3

[a]Abbreviations: MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.
[b]Values are means ± SD for four experiments.
[c]Standard deviations as given for 18:0 and 10:0.

TABLE 2

Calculation of the Fatty Acid Composition (mol%) of the *sn*-1(3) Positions of 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) from the Composition of Other Acylglycerols Obtained by Deacylation with Allyl Magnesium Bromide[a]

Formulas for calculation	SOS 1(3)-MAG % 18:0[a,b]	CPC 1(3)-MAG %10:0[a,b]
1(3)-MAG (from Table 1)	93.2 ± 3.8	92.3 ± 1.4
1.5 × TAG − 0.5 × 2-MAG[c]	98.8 ± 0.7	99.4 ± 1.4
2 × 1,2(2,3)-DAG − 2-MAG	105.5 ± 7.2	102.1 ± 2.4
1,3-DAG (from Table 1)	98.3 ± 1.7	93.8 ± 2.1
3 × TAG − 2 × 1,2(2,3)-DAG	92.1 ± 7.3	96.7 ± 3.1

[a]Abbreviations: See Table 1.
[b]Values are means ± SD for four experiments.
[c]The best estimate for the fatty acid composition of the *sn*-1(*sn*-3) positions of the triacylglycerols.

TABLE 3

Calculation of the Fatty Acid Composition (mol%) of the *sn*-2 Positions of 1,3-Distearoyl-2-oleoyl-glycerol (99% +) (SOS), and 1,3-Didecanoyl-2-palmitoyl-glycerol (97% +) (CPC) from the Composition of Other Acylglycerols Obtained by Deacylation with Allyl Magnesium Bromide[a]

Formulas for calculation	SOS 2-MAG % 18:0[b]	CPC 2-MAG %10:0[b]
2-MAG (from Table 1)[c]	2.4 ± 0.8	1.2 ± 1.3
4 × 1,2(2,3)-DAG − 3 × TAG	15.7 ± 14.4	6.6 ± 4.7
3 × TAG−2 × 1,3-DAG	3.4 ± 3.7	12.5 ± 4.9
3 × TAG−2 × 1(3)-MAG	13.7 ± 7.7	15.4 ± 3.7
2 × 1,2(2,3)-DAG − 1(3)-MAG	14.7 ± 8.1	11.0 ± 2.5
2 × 1,2(2,3)-DAG − 1,3-DAG	9.6 ± 7.4	9.5 ± 2.5

[a]Abbreviations: See Table 1.
[b]Values are means ± SD for four experiments.
[c]The best estimate for the fatty acid composition of the *sn*-2 positions of the triacylglycerols.

sn-1(*sn*-3) and *sn*-2 is estimated from the composition of TAG and 1,2(2,3)-DAG. The equations employed are:

$$1(3)\text{-MAG} = 3 \times \text{TAG} - 2 \times 1,2(2,3)\text{-DAG} \quad [1]$$

$$2\text{-MAG} = 4 \times 1,2(2,3)\text{-DAG} - 3 \times \text{TAG (6)} \quad [2]$$

The results obtained with these equations are shown in Tables 2 and 3. The equations suggested for the EMB method showed larger standard deviations than the equations used with the AMB method due to larger factors and, in the case of 2-MAG, more terms. Furthermore, the results from the AMB method were closer to the theoretical value.

The possible discrimination between the primary and secondary positions in TAG by the present method was also examined, using 1,2,3-tripalmitoylglycerol. With 7% of the tripalmitin degraded, the ratio between 1(3)-MAG and 2-MAG, and between 1,2(2,3)-DAG and 1,3-DAG, respectively, approached the theoretical value of 2. This means that the positional discrimination was of minor importance, at least for palmitic acid. However, the calculations are based on mole% of fatty acid in each class of acylglycerol, and not the amount of these; therefore a certain positional discrimination will not influence the results obtained with the present method.

The amount of AMB added is theoretically enough to deacylate all ester bonds of about 250 mg of TAG. That only a minor proportion of the TAG was deacylated is partly due to the content of azeotropic water in ether. The amount of water should be minimized as it increases the polarity of the solvent, and hence, in principle, the rate of acyl migration. Therefore, an excess of AMB must be added to compensate for the reaction with the water present. This reaction produces magnesium salts which further increases the polarity of the solvent. In order to minimize isomerization, boric acid is added to the washing solutions (11).

In conclusion, the method presented offers a fast and simple way to carry out regiospecific analyses of triacylglycerols. Sample sizes of about 6 mg were used, but smaller amounts (2 mg) can also be analyzed. This makes the method well suited for biological samples. The method can be used for most fats, including those for which the pancreatic lipase assay is unreliable. The results obtained are also more accurate and have smaller standard deviations than when EMB is used. In our laboratory the AMB method has proven to be particularly useful for the analysis of triacylglycerols, such as marine oils, that contain long-chain polyunsaturated fatty acids and for seed oils that contain medium-chain fatty acids (C_8–C_{12}).

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Microplate Methods for Determination of Serum Cholesterol, High Density Lipoprotein Cholesterol, Triglyceride and Apolipoproteins

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Microtiter plate methods were developed for the enzymatic determination of serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG), and for the turbidometric determination of apolipoproteins. The micromethods resulted in accurate, precise values that were in good agreement with the conventional spectrophotometric assays. The coefficient of variation for TC determinations was 4.5% or less and bias was 5% or less. The lipid micromethod assays are sensitive to 10 mg/dL or less, and the apolipoprotein assay to 1 mg/dL. Less than 100 μL of serum suffices for TC, TG and apoprotein assays; HDL-C requires an additional 100 μL of serum. Advantages of the micromethods include reductions in assay time and in the amount of reagents required.
Lipids 28, 151-155 (1993).

In studies requiring large numbers of samples for assay of serum lipids and apoproteins in duplicate or triplicate, time and cost of the assays become significant factors. In both clinical and research laboratories, serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglyceride (TG) assays are most often performed using "test sets or kits" supplied by commercial sources. The methods recommended by the National Cholesterol Education Program (NCEP) (1) are enzymatic assays which may be done by either manual or autoanalyzer procedures (2,3). Apolipoprotein quantification usually involves either antibody-based or other types of precipitation assays (4,5), electroimmunoassay (6) or immunonepholometric assays (7).

The advantages of micromethods for determination of serum lipid levels include the small sample size required and a great savings in terms of time and cost of reagents and reference standards. This paper describes the adaption of enzymatic and turbidometric "test set" methods to microprocedures for determination of serum TC, HDL-C, TG and apolipoproteins A-I, A-II and B.

MATERIALS AND METHODS

Total serum cholesterol. The test set (Procedure No. 1010) was purchased from Stanbio Laboratory, Inc. (San Antonio, TX). It is based on the principles of the procedures defined by Allain *et al.* (2) and Trinder (3). The standard procedure using this commercial test set involves mixing 1.0 mL reagent with 10 μL standard supplied with the test set, 10 μL serum, or 10 μL phosphate-buffered saline (PBS) for the blank, and incubating for 15 min at room temperature before reading the absorbance against the blank in a Beckman (Palo Alto, CA) DU-40 spectrophotometer. For comparison to the plate reader in the microassay, absorbance was read at 490 nm. Calculations were performed as described in the test set. To determine the validity of the microtiter plate method, dilutions of serum and standard with PBS were assayed with both the spectrophotometer and the microtiter plate reader, using 50 μL of the 1:5 diluted sample and 1 mL reagent. The cholesterol standard (200 mg/dL) was diluted 1:5 with the same solvent (ethyleneglycol monomethyl ether) in which the original standard was dissolved.

For the micromethod, 100 μL of the Stanbio cholesterol reagent were added to wells containing 10 μL of PBS, 10 μL 1:5 diluted calibration standards, 10 μL 1:5 diluted reference sera or 10 μL 1:5 diluted fresh human serum in ELISA plates (Corning Co., Corning, NY). Samples were run in sets of 3-5 using 2 separate dilutions. Reference sera, including both normal and elevated lipid levels, were obtained from the National Bureau of Standards (NBS; Gaithersburg, MD) and Fisher Diagnostics (Orangeburg, NJ). After mixing by shaking the plate gently and incubating at room temperature for 15 min, bubbles were eliminated with a gentle stream of nitrogen. Absorbance was read at 490 nm in a microplate reader with an automix feature (UVmax™, Molecular Devices Corp., Menlo Park, CA). The mean absorbance from 5 PBS blanks was subtracted from each reading to provide the final reading.

To determine whether the use of a single standard concentration was as accurate as the use of a calibration curve, the latter was constructed by dilution of the cholesterol standard (200 mg/dL) to concentrations of 80, 60, 40, 30, 20 and 10 mg/dL. Absorbance values of samples were used to calculate and compare concentrations derived from the linear regression of the calibration line to those calculated by using only the 40 mg/dL concentration.

HDL-Cholesterol. The commercial method was performed as outlined in the brochure accompanying the test kit and compared to the micromethod. The reagent is the same as that used in the TC assay. Stanbio HDL-Cholesterol Procedure No. 0599 was used. Five minutes after mixing 0.5 mL serum with 50 μL precipitating reagent (1M $MgCl_2$ in aqueous 1% dextran sulfate, M_r = 500,000 D) (8), samples were centrifuged for 10 min in a microcentrifuge at 11000 rpm. Fifty μL of the clear supernatant were mixed with 1.0 mL cholesterol reagent and treated as described above for TC.

For the micromethod, 100 μL serum samples or reference sera were mixed with 10 μL precipitating reagent and centrifuged as described above; 100 μL enzymatic reagent were added to 5 μL aliquots of the supernatant in the plate wells and handled as described for TC. Two separate precipitations were done per serum sample. Five μL aliquots of the diluted standard cholesterol solution (40 mg/dL) were run in 5 wells and 5 μL PBS were used in each blank.

Triglycerides. The Stanbio Enzymatic Triglycerides Procedure No. 2000 is a colorimetric method based on a procedure (9) involving hydrolysis of triglyceride and subsequent enzymatic phosphorylation and oxidation of the

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Abbreviations: apo, apolipoprotein; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; HDL-C, high density lipoprotein cholesterol; NBS, National Bureau of Standards; NCEP, National Cholesterol Education Program; PBS, phosphate-buffered saline; std, standard; TC, total cholesterol; TG, triglyceride.

liberated glycerol. The method was performed as outlined in the brochure accompanying the test kit, except that absorbance was read at 490 rather than 500 nm. The procedure is essentially identical to that for TC, using 10 μL of the standard, reference or sample sera and 1 mL reagent. For the microprocedure, 100 μL reagent were mixed with 10 μL of PBS, diluted standard, diluted reference or diluted sample sera in the plate wells and absorbance was read at 490 nm after 15 min. Values for samples and references were compared using a single standard (40 mg/dL) with those calculated from the linear reqression of the calibration curve.

Apolipoproteins. Test sets for the turbidometric determination of apolipoproteins A-I, A-II and B (apo A-I, apo A-II, apo B) were purchased from Boehringer Mannheim Biochemica (Indianapolis, IN). The assay is based on the spectrophotometric determination of the turbidity of the antigen-antibody reaction. It is essentially the same for each apoprotein, except for the specific antiserum. Briefly, antiserum was diluted 10-fold with the antiserum diluent as specified by the manufacturer. Samples and calibration sera were diluted with PBS. In the conventional method, 50 μL diluted serum or standard were added to 1.0 mL diluted antiserum, mixed and incubated at 25°C for 2.5 h. The test set procedure specifies absorbance reading at 365 nm, but this wavelength was unavailable in the microplate reader. As the spectrophotometric absorbances of standards at 405 and 365 nm were essentially identical, microplate reader absorbance was read at 405 nm.

In the micromethod, 5 μL of diluted calibration serum, serum samples or PBS were each combined in plate wells with 100 μL diluted antiserum and incubated at 25°C for 2.5 h. The calibration serum was diluted 10- to 80-fold for construction of the apo A-I standard curve and 5- to 40-fold for apo A-II and apo B. Sample and reference sera were diluted 1:10 for apo B and apo A-II and 1:20 for apo A-I determinations. Absorbance was determined at 405 nm in the microplate reader; the mean absorbance of five blanks was subtracted from the sample values. The concentrations of diluted samples were determined from the calibration curves, and these values were multiplied by the dilution factor in the final calculation.

Statistical analyses on calibration curves and replicate comparisons were by analysis of variance using the procedures of Minitab (copyright, Penn State Univ., PA) in the VAX computer.

RESULTS

Absorbance readings for TC, HDL-C and TG resulted in very similar values for each comparison when samples were prepared by the conventional test set procedure and read first in the spectrophotometer and then in the microplate reader. Preliminary experiments showed that a total volume of 110 μL per well in the microtiter plate was sufficient for accuracy. A comparison of the same samples indicated that there were no significant differences between cholesterol values obtained using 220 μL total volume *vs.* 110 μL (97 ± 2 mg/dL *vs.* 93 ± 4, respectively, n = 6). Therefore, 100 μL reagent and 10 μL diluted serum were used in the final recommended procedure.

Calibration curves developed for total cholesterol from representative assays are shown in Figure 1. Essentially

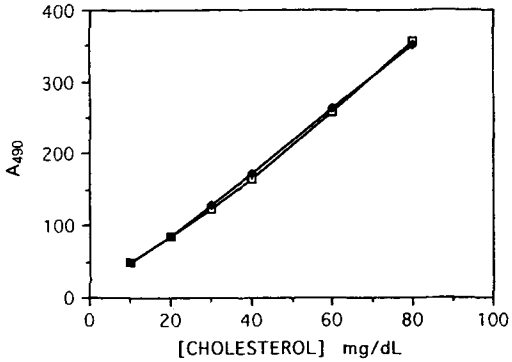

FIG. 1. Two typical cholesterol calibration curves. Two separate dilutions of a cholesterol standard were made as indicated in Materials and Methods and assayed by the micromethod. Absorbance was determined at 490 nm in a microplate reader. Five determinations provided the mean value for each point.

identical curves resulted from two replicates with five determinations each of a calibration standard (200 mg/dL) diluted on two separate occasions to 10, 20, 30, 40, 60 and 80 mg/dL. Precision was good as the intra-assay coefficient of variation (CV) for these values averaged 1.4% (range 1.0–1.6%), while the inter-assay CV was 2.8% (range 2.0–3.9%).

Similar values for each lipid class were obtained when comparing samples assayed by the conventional test set procedure to the same samples assayed by the micromethod (Table 1). The repeatability of the TC values from reference sera was good. The micromethod CV shown in Table 1 represents both inter- and intra-assay variation, as there were four determinations in each of two microassays; it ranged from 1–4.5%. The accuracy of the TC values, based on the "true" concentration given by the suppliers of the reference sera, ranged from 3.4–5%. In some assays, the conventional method provided slightly more accurate values; however, these were not significantly different from micromethod values ($P > 0.05$). Although there was good agreement between the values for HDL-C from both methods, the CV and bias for the micromethod were relatively large, up to 7.2 and 10.5%, respectively (Table 1). Likewise, there was no significant difference between the methods for TG, but deviations from the given "true" values ranged from 2–10%.

TC, HDL-C and TG values calculated from calibration curves were very similar to the values derived by using the absorbance of a single calibration standard (40 mg/dL) (Table 2). No significant differences were found between the mean values from the two methods. Because the calibration curve requires a number of plate wells, but offers no great advantage in accuracy of results, a single standard can be recommended.

The spectrophotometric assay for apoproteins resulted in the same value when the standard curve and sample were read at either 365 or 405 nm in the spectrophotometer. The values determined in the microplate reader at 405 nm for these conventional preparations were similar, and it was concluded that it was feasible to use the microplate reader (Table 3). Also, standards and samples prepared by the micromethod resulted in comparable values for apo B (Table 3), as well as for apo A-I and A-II (data

TABLE 1

Comparison of Conventional and Microplate Lipid Assays[a]

	Micromethod[b]	Conventional[c]	Reference[d]	CV (%)[e]	Bias (%)[f]
		Total cholesterol (mg/dl)[h]			
Serum[g]					
A	178 ± 3	179	172 ± 28	1.0	3.4
B	298 ± 8	304	309 ± 48	2.2	3.6
C	136 ± 8	140	141 ± 5	4.5	5.0
		HDL-C (mg/dL)[i]			
A	44 ± 4	41	48 ± 9	6.0	3.5
B	96 ± 8	91	87 ± 21	7.2	10.5
C	23 ± 2	22	—	7.0	—
		Triglyceride (mg/dL)[j]			
A	89 ± 4	92	100 ± 15	3.4	11.0
B	260 ± 7	253	261 ± 52	2.2	2.2
C	48 ± 2	53	—	3.7	—

[a]Each serum sample was diluted twice for two separate assays by each method.
[b]Values represent the mean ± SD of eight determinations by the microplate reader.
[c]Values represent the mean of four determinations (duplicates of two dilutions) in the spectrophotometer.
[d]Values were furnished by the suppliers of the reference sera.
[e]The coefficient of variation is a measure of the reproduciblity of the microassay.
[f]Bias is a measure of the accuracy of the microassay (variation from the true value).
[g]Serum A is a normal lipid level reference serum and serum B an elevated lipid level reference serum supplied by Fisher Diagnostics (Cinncinati, OH). Serum C is a normal level reference serum supplied by the National Bureau of Standards.
[h]For the microassay cholesterol concentration was calculated: Total Cholesterol (mg/dL) = $A_{sample}/A_{std} \times 40$ mg/dL $\times$ 5. The concentration of the diluted standard was 40 mg/dL; the factor 5 corrects for the 1:5 dilution of the serum sample. Concentration for the conventional method was calculated as specified in the test set: Total Cholesterol (mg/dL) = $A_{sample}/A_{std} \times$ [standard]. Std, standard.
[i]Microassay high density lipoprotein cholesterol = (HDL-C) values were calculated from the equation: HDL-C (mg/dL) = $A_{sample}/A_{std} \times 40$ mg/dL $\times$ 1.1, where 1.1 is the dilution factor for the precipitation step. Concentration for the conventional method was calculated as specified in the test set.
[j]Microassay TG values were calculated from the equation: Triglycerides (mg/dL) = $A_{sample}/A_{std} \times 40$ mg/dL $\times$ 5, where 40 is the concentration of the diluted standard and 5 is the factor for dilution of the sample. Concentration for the conventional method was calculated as specified in the test set.

not shown). Standard curves developed for the apoproteins are shown in Figure 2. Intra-assay variation was very small, but the inter-assay CV averaged approximately 4%. Values calculated from single standard determinations were not as satisfactory as those obtained from standard curves.

DISCUSSION

The TC, HDL-C, TG and apoprotein microassays described are sensitive, reliable, economical and rapid. The reagent for TC and HDL-C assays contains a combination of enzymes (peroxidase, cholesterol esterase and oxidase) and a chromogen. The intensity of the final color is proportional to the cholesterol concentration. The calibration curve was found to be linear to concentrations of 10 mg/dL for both cholesterol and TG. Even though a microplate reader is an expensive instrument, a 96 well plate can be read in approximately one minute. If three wells are used to provide the mean blank value, three for the standard, and three each for reference sera (normal and elevated lipids) for TC and HDL-C, the 78 remaining wells can accommodate 13 serum samples for TC and HDL-C in triplicate or 19 samples of each in duplicate. When large numbers of samples must be processed, it would be most economical to process TC and HDL-C together and triglycerides on a separate plate, as the blanks, standards and reference sera should be determined on each plate.

The dilution of standards and sera is the most critical part of the microassay as very small volumes are involved. The use of reference sera for TC and TG is strongly recommended for both research and clinical laboratories by the NCEP so that accuracy can be assessed (1). The NCEP has established criteria to minimize the CV and bias of TC assays with the hope that these can be reduced to 3% or less. In the present studies, the microassay values approached this goal with one set of reference standards, but we cannot explain why there was consistently greater bias (5%) with the reference serum from NBS. Interim recommendations of the NCEP Coordinating Committee have set bias for HDL-C at 10% or less and CV at 6% or less (10); these have been revised upwards from those criteria set in 1988 (1), because those goals were unrealistic given the current methods for HDL-C determinations. The CV and bias for HDL-C in the present study approached those now recommended. The NCEP

TABLE 2

Comparison of Standard Curve *vs.* Single Standard Values in the Lipid Microassays[a]

Sample	Total cholesterol (mg/dL)		HDL-C (mg/dL)		Triglyceride (mg/dL)	
	Std curve[b]	Calculated[c]	Std curve	Calculated	Std curve	Calculated
Reference serum A[d]						
Plate 1	171	178 ± 7	40	40 ± 2	92	89 ± 3
Plate 2	175	178 ± 5	40	41 ± 1	95	93 ± 4
Reference serum B[e]						
Plate 1	280	295 ± 7	87	90 ± 4	265	268 ± 10
Plate 2	289	294 ± 3	88	89 ± 4	271	267 ± 10
Sample[f]						
Plate 1	212	222 ± 7	—	—	—	—
Plate 2	219	222 ± 4	—	—	—	—

[a]Total cholesterol values (mean ± SD) are based on eight determinations; two dilutions were used. High density lipoprotein cholesterol (HDL-C) values are based on 10 determinations; two precipitations were used. Triglyceride concentrations are based on 10 determinations, using two dilutions. Std, standard.
[b]Values were calculated from the regression equation of the standard curves.
[c]Concentrations were calculated according to the equation shown in Table 1, using a single standard.
[d]The supplier of this reference serum for normal lipid values was Fisher Diagnostics (Cinncinati, OH).
[e]Reference serum for elevated lipid values was supplied by Fisher Diagnostics.
[f]Fresh human serum obtained from the local blood bank.

TABLE 3

Comparison of Apoprotein B Determinations by Conventional and Microturbidometric Assays

ApoB (mg/dL)			
Conventional[a]		Microassay[b]	
A_{365}	78.0	A_{405} [1]	76 ± 3.4
A_{405}	78.5	A_{405} [2]	72 ± 2.5
		A_{405} [3]	75 ± 1.5

[a]Duplicate samples in a representative assay read in the spectrophotometer at both 365 and 405 nm.
[b]Mean ± SD of the same sample in a titer plate. Value for sample A_{405} [1] is the mean ± SD of values from four wells in which 5 μL diluted serum and 100 μL antiserum were mixed. A_{405} [2] represents the mean value of the conventional samples shown in the left column when the aliquots were dispensed into four wells. A_{405} [3] is the value obtained on four wells containing the same sample as [1] assayed on the following day with new dilutions of sample and standards.

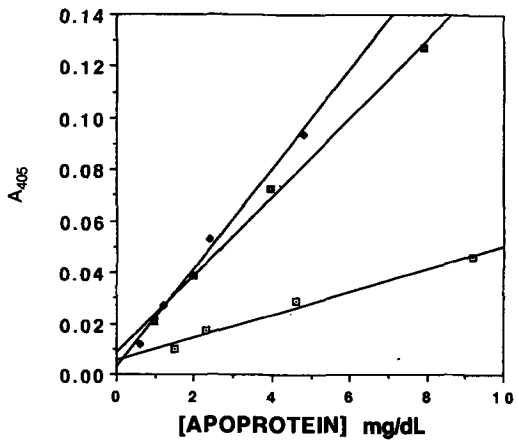

FIG. 2. Apoprotein calibration curves in a representative assay. The dilutions of both antisera and individual apoprotein calibration standards are described in Materials and Methods. Each point represents the mean of samples from duplicate microtiter plates: (open box with centered dot) apo A-II; (closed box) apo A-I; (closed box with centered dot) apo B.

has set 5% as the acceptable CV and bias for TG, thus our value of 11% bias using the Fisher Diagnostic normal reference does not meet the criterion. The values were well within the range of values stated by this supplier, however, and we attribute the large bias to the fact that the "true" value is based on a mean value determined from a number of different methods.

Several observations aided in developing these assays. Better results were obtained when the standard or serum sample was dispensed first on the bottom wall of the well before the addition of the 100 μL reagent. We did not find it necessary or more efficient to pretreat the wells with Tween 20, as reported by Belcher *et al.* (11) in their micromethod for lipid measurements in lipoprotein subfractions. The color of the TG sample is very stable and supplies identical values if read between 15 and 50 min. The TC and HDL-C microtiter plates should be read between 15 and 30 min. The micromethod using the turbidometric assay for apoproteins is not as sensitive as ELISA methods, but its sensitivity allows quantification in the range of a few mg per dL. Unlike the lipid assays, it seems advisable to use calibration curves for all apolipoprotein assays. The curve is not linear at concentrations higher than those used here; therefore if the absorbance of a sample is off the curve, the sample should be further diluted and reassayed. It is recommended by the manufacturer of the apoprotein test materials that each laboratory repeat the assay on two or more serum samples until a normal range of bias is established for the method.

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COMMUNICATION

A Lack of Correlation Among Fatty Acids Associated with Different Lipid Classes in Human Milk

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The fatty acids associated with triacylglycerol, cholesteryl ester, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin in human milk were compared. Ten milk samples were selected for lipid class analysis based on their total lipid polyunsaturated/saturated fatty acid ratio (P/S ratio). The P/S ratio of the selected milk samples ranged from 0.3 to 0.8. Linoleic acid was the predominant fatty acid in milk to affect the P/S ratio. The percentage of linoleic acid in milk triacylglycerol was correlated ($r = 0.84$, $P < 0.05$) with the total milk lipid P/S ratio. Linoleic acid esterified to cholesterol was not correlated with total milk lipid P/S ratio but was correlated ($r = -0.66$, $P < 0.05$) with the quantity of lipid in the milk. Linoleic acid in the phospholipid classes did not correlate with shift in P/S ratio of the total milk lipid or linoleic acid content of other lipid classes.
Lipids 28, 157–159 (1993).

Lactating human mammary tissue secretes between 25 and 50 g of milk lipid per day. The major lipid classes in milk, which appear to remain proportionately constant, are the triacylglycerol (*ca.* 98%), phospholipids (*ca.* 1%) and cholesterol/cholesteryl esters (*ca.* 0.5%) (1). Although the phospholipids and cholesteryl esters are minor components of milk, they contain significant amounts of the long-chain unsaturated fatty acids required by the infant for synthesis of eicosanoids and structural lipids (2). These lipid classes are digested and absorbed differently than the milk triacylglycerols, and their fatty acids may have a different metabolic fate than the fatty acids from the triacylglycerols.

The purpose of this study was to make within-sample comparisons of the fatty acids associated with the major lipid classes in human milk. The fatty acids associated with the major lipid classes in breast milk have been identified and the literature recently reviewed by Jensen (3). Whereas most reports provide data on a limited number of lipid classes, a series of studies by Bitman *et al.* (4–6) provide information on fatty acids associated with the major lipid classes in breast milk from a group of mothers delivering prematurely and at full-term. From this literature it is known that there is a variation in fatty acids associated with all the lipid classes in breast milk. However, it is impossible to determine from the literature if there is any correlation between changes in fatty acids among the lipid classes. By comparing fatty acid composition of lipid classes within a milk sample, it is possible to determine how fatty acids associated with different lipid classes vary relative to each other.

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Abbreviations: GLC, gas-liquid chromatography; P/S ratio, ratio of polyunsaturated to saturated fatty acids; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Ten milk samples containing a wide range of unsaturated fatty acids were chosen for this study from an initial screening of approximately a hundred milk samples. The samples were complete breast expressions donated by mothers who had delivered full-term infants and had been lactating for at least a month. The general protocol for collection and subsequent handling of samples has been described (7).

On the day of analysis the milk samples were thawed to 38°C, homogenized, and a 15-mL aliquot was taken. The lipids then were extracted by a modified Folch procedure (8). Total lipid was determined by weighing the lipid extract. Preliminary separation of the polar and nonpolar milk lipids was on a column containing Unisil (Clarkson Chemical Co., Williamsport, PA) as recommended by Rouser *et al.* (9). The triacylglycerols and cholesteryl esters in the nonpolar fraction were isolated on silica gel G thin-layer chromatographic plates developed with hexane/diethyl ether/glacial acetic acid (90:30:2, by vol). When necessary, further purification of cholesteryl esters and triacylglycerols was achieved by a second plating on silica gel G plates developed with hexane/diethyl ether (95:5, vol/vol).

An aliquot of the triacylglycerols was used for structural analysis. The fatty acids esterified to the primary (*sn*-1,3) and secondary (*sn*-2) positions of the triacylglycerol were determined by digestion with pancreatic lipase (10). The residual triacylglycerols and *sn*-2 monoglycerols were isolated from the digestion mixture by thin-layer chromatography (TLC) on silica gel G plates containing 5% boric acid developed in chloroform/methanol (96:4, vol/vol).

The polar lipids were separated by the use of two TLC systems. First, the polar lipids were separated on silica gel G plates developed in chloroform/methanol/water (75:30:3, by vol). Phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were eluted from the silica gel G and complete isolation obtained by a second plating on silica gel H. The second plating was developed in chloroform/methanol/acetic acid/water (25:15:4:2, by vol).

The fatty acids associated with triacylglycerol, *sn*-2 monoacylglycerol and cholesteryl esters were prepared for gas-liquid chromatography (GLC) by transmethylation with 2.5 N sodium methoxide (11). The fatty acids associated with the total polar lipids, sphingomyelin, phosphatidylcholine and phosphatidylethanolamine were transesterified in methanol/benzene (4:1, vol/vol) with acetyl chloride (12).

The methyl esters of fatty acids were separated by GLC on a Supelcowax 10 fused capillary column, 30 m × 0.53 mm i.d. (Supelco, Bellefonte, PA). Identification of fatty acids was based on the retention times of known fatty acids.

Statistical analyses included Pearson's correlation analysis and linear regression analysis among the fatty

acids in the different fractions and total milk polyunsaturated/saturated fatty acid ratio (P/S ratio) (13).

RESULTS AND DISCUSSION

The proportions of fatty acids in the three major phospholipid classes are shown in Table 1. There are relatively few values available on the fatty acid composition of phospholipids in breast milk, and the values in Table 1 are in general agreement with the literature (5,14). The range of unsaturated fatty acids in the total phospholipid fraction was 42–56%, with linoleic acid (*ca.* 22%) being the major polyunsaturated fatty acid (PUFA). A large proportion of the fatty acids were unsaturated in phosphatidylcholine (*ca.* 52%) and phosphatidylethanolamine (*ca.* 61%), and only 19% of the fatty acids in sphingomyelin were unsaturated. As previously reported, the major unsaturated fatty acid in sphingomyelin was nervonic acid (24:1), a fatty acid not observed in the other lipid classes (5). Linoleic acid was approximately 2% in sphingomyelin and arachidonic acid was not detected. The unsaturated fatty acids in the phospholipid classes were relatively constant and did not correlate with proportions of unsaturated fatty acids in the other major lipid classes in milk.

The total unsaturated fatty acids in the neutral lipid fraction containing cholesteryl esters and triacylglycerols had a range of 50–65%. The unsaturated fatty acids were 60% in triacylglycerols and 55% in cholesteryl esters (Table 2). Based on the literature, it appears that the fatty acids esterified to cholesterol in human milk are quite variable.

In previous reports, fatty acids of cholesteryl esters were reported to be more saturated (15,16), similar to (17,18) or less saturated (6) than the fatty acids of triacylglycerols. The primary difference in these reports was the values for linoleic acid esterified to cholesterol. Average

TABLE 1

Fatty Acid Composition of Phospholipids in Human Milk (wt%)[a]

Fatty acid	Total polar lipids	Sphingomyelin	Phosphatidylcholine	Phosphatidylethanolamine
12:0	0.80 ± 0.38	0.40 ± 0.29	0.70 ± 0.43	0.75 ± 0.29
14:0	2.60 ± 0.89	1.68 ± 0.30	2.01 ± 1.10	2.30 ± 0.79
16:0	13.80 ± 2.89	9.42 ± 1.62	22.78 ± 2.99	10.93 ± 3.07
16:1	1.68 ± 0.75	0.35 ± 0.20	2.87 ± 1.05	2.74 ± 1.07
18:0	21.63 ± 2.70	11.14 ± 2.48	22.60 ± 3.13	24.18 ± 2.23
18:1	17.69 ± 1.20	4.05 ± 1.14	16.15 ± 1.30	23.97 ± 2.29
18:2	22.05 ± 1.39	2.15 ± 0.56	26.50 ± 3.33	24.82 ± 2.72
18:3	0.80 ± 0.28	—	0.37 ± 0.14	1.00 ± 0.45
20:0	1.48 ± 0.50	7.19 ± 1.48	0.20 ± 0.18	0.20 ± 0.15
20:3	—[b]	—	1.19 ± 0.31	1.69 ± 0.29
20:4	4.10 ± 1.12	—	3.09 ± 1.25	6.49 ± 2.23
21:0	0.31 ± 0.18	0.70 ± 0.25	0.18 ± 0.15	0.25 ± 0.20
22:0	5.90 ± 1.10	20.79 ± 2.32	—	—
22:1	—	2.33 ± 1.74	—	—
22:4	0.50 ± 0.28	—	0.20 ± 0.15	1.23 ± 1.03
22:5	0.38 ± 0.15	—	0.36 ± 0.35	0.58 ± 0.34
22:6	0.80 ± 0.20	—	0.37 ± 0.32	2.00 ± 0.58
23:0	0.93 ± 0.56	4.01 ± 1.54	—	—
24:0	3.89 ± 1.00	14.96 ± 5.01	—	—
24:1	2.25 ± 0.89	10.30 ± 3.93	—	—

[a]Results are mean of 10 observations ± SD.
[b]Fatty acid value less than 0.15 wt%.

TABLE 2

Comparison of Fatty Acids Esterified to Cholesteryl Ester and Triacylglycerol in Human Milk (wt%)[a]

Fatty acid	Cholesteryl ester	Triacylglycerol	
		Total	*sn*-2 Position[b]
12:0	1.57 ± 0.77	4.28 ± 1.08	2.88 ± 1.12
14:0	4.85 ± 2.07	5.87 ± 1.87	8.26 ± 1.90
16:0	23.64 ± 3.37	20.65 ± 3.61	47.85 ± 4.67
16:1	5.40 ± 1.92	3.01 ± 0.95	1.23 ± 1.00
18:0	9.30 ± 1.60	7.09 ± 0.73	2.86 ± 0.96
18:1	26.24 ± 9.85	34.59 ± 3.01	17.78 ± 3.65
18:2	20.49 ± 6.30	20.20 ± 4.93	13.79 ± 3.83
18:3	0.67 ± 0.34	1.39 ± 0.45	0.74 ± 0.39
20:4	1.33 ± 0.68	0.55 ± 0.09	1.46 ± 0.895
22:6	0.42 ± 0.32	0.21 ± 0.12	0.45 ± 0.19

[a]Results are mean of 10 observations ± SD.
[b]Determined by digestion of triacylglycerol with pancreatic lipase.

cholesteryl linoleate in milk has been reported as 6.2% (16), 6.9% (15), 12.4% (17), 19.4% (18) and 30.6% (6). In this report, linoleic acid esterified to cholesterol ranged from approximately 9–30%, which encompasses most of the values from previous reports. The linoleic acid content of milk triacylglycerol ranged from 14 to 27%, which is not quite as great as that observed with the cholesteryl esters.

The fatty acids esterified to the *sn*-2 position of the milk triacylglycerol are presented in Table 2. Fatty acids are not randomly distributed among the positions of triacylglycerol. As observed in previous reports, 16:0 is found predominantly in the *sn*-2 position of the milk triacylglycerol, and the unsaturated fatty acids, 16:1, 18:1, 18:2 and 18:3, are found predominantly in the *sn*-1,3 position (14,19,20). Unlike the other unsaturated fatty acids, arachidonic acid and docosahexaenoic acid are also found predominantly in the *sn*-2 position. The predominance of arachidonic acid in the *sn*-2 position supports a previous report (20). The observation with docosahexaenoic acid, to our knowledge, has not been reported. However, this observation should be viewed with some caution as pancreatic lipase digestion was used for positional analysis and there is some debate about the relative activity of pancreatic lipase toward docosahexaenoic acid (21,22).

The total lipid in the milk samples used in this study ranged from 1.5 to 8.1 g/dL with a mean of 3.8 ± 2.4 g/dL SD. The samples had a P/S ratio that ranged from 0.3 to 0.8. Linoleic acid in the milk was the predominant fatty acid to affect the P/S ratio. When linoleic acid in the individual lipid classes is compared to the P/S ratio of the total milk lipid, different relationships are observed. As expected, linoleic acid content of milk triacylglycerol was correlated ($r = 0.84$, $P < 0.05$) with the P/S ratio of the total milk lipid. Linoleic acid content in both the primary and the *sn*-2 positions increase with the P/S ratio. The linoleic acid associated with the phospholipid [$r = -0.16$, (nonsignificant)] and cholesteryl ester ($r = 0.26$, ns) were not significantly correlated with whole milk P/S ratio. Rather than being related to the composition of fatty acids in the milk, the amount of linoleic acid esterified to cholesterol appears to be negatively correlated ($r = -0.66$, $P < 0.05$) with the quantity of lipid in the milk.

In this study, the milk phospholipids and cholesteryl esters contained substantial amounts of unsaturated fatty acids, but the fatty acids esterified to these lipid classes are not correlated with each other or with the amounts found in milk triacylglycerol. The phospholipids are part of the fat globule membranes in milk, and their composition appears to be closely maintained. The distribution of fatty acids esterified to cholesterol is more variable than observed with the phospholipids, and it may be affected by total milk lipid synthesis. The enzyme responsible for the esterification of fatty acids to cholesterol in milk has not been identified. The fatty acids esterified to the milk triacylglycerol make up the bulk of the fat globule core and may be most responsive to changes in the amounts of available fatty acids. This is supported by a previous observation that deuterium-labeled linoleic acid is incorporated into breast milk triacylglycerol, cholesteryl ester and phospholipid, but the influence of available linoleic acid on milk triacylglycerol is much greater than on milk cholesteryl esters and phospholipid (23).

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Synthesis of Trisubstituted C_{18} Pyrrole Fatty Ester Derivatives

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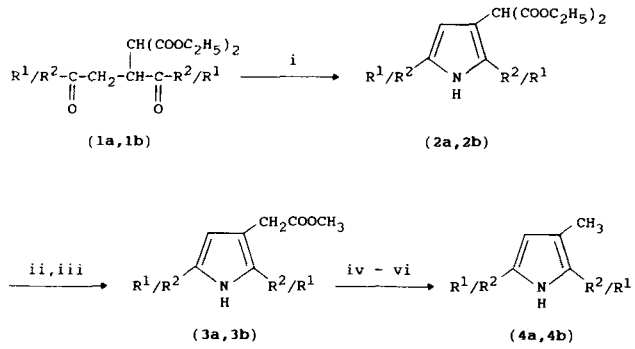
Reaction of methyl 10(11)-dicarbethoxymethyl-9,12-dioxooctadecanoate (1a,1b) with ammonium acetate furnished a mixture of positional isomers of a pyrrole derivative, methyl 9,12-imino-10(11)-dicarbethoxymethyl-9,11-octadecadienoate (2a,2b). Decarboxylation of the mixture of compounds 2a,2b with sodium carbonate in aqueous methanol yielded a mixture of compounds 3a,3b containing a CH_2COOCH_3 group at the 3- or 4-position of the pyrrole ring after esterification. Heating of the hydrolyzed mixture of compounds 3a,3b at 180°C for 1 h gave the desired trisubstituted pyrrole derivatives, methyl 9,12-imino-10(11)-methyl-9,11-octadecadienoate (4a,4b), containing a methyl group at the 3- or 4-position of the pyrrole nucleus. The structures of the products and intermediates were confirmed by infrared, and by ^1H and ^{13}C nuclear magnetic resonance spectroscopy.
Lipids 28, 161–162 (1993).



where $R^1 = CH_3(CH_2)_5$; $R^2 = (CH_2)_7COOCH_3$; i) $(NH_4)_2CO_3$, AcOH; ii) Na_2CO_3, MeOH, H_2O; iii) BF_3/MeOH; iv) KOH, EtOH; v) HCl; vi) 180°C, 1 h.

SCHEME 1

We have recently reported a new synthetic approach for the introduction of a methyl group into the furan ring of a 2,5-disubstituted C_{18} furanoid fatty ester *via* a malonic acid function (1). The mechanism of the conversion of the CH_2COOCH_3 substituent to a CH_3 group, located at the 3- or 4-position of the furan ring in one of the intermediates, was postulated to be analogous to the decarboxylation process of α,β-unsaturated carboxylic acids (2). In continuing our study of this novel type of reaction, we have extended our investigation to include a pyrrole system. Fatty acid derivatives containing a pyrrole or *N*-methylpyrrole nucleus were obtained during the oxidative cleavage of the furan ring of furanoid fatty acids by lipoxygenase present in bovine liver homogenate when conducted at pH 7.5 and in using cofactors known to stimulate fatty acid oxidation (3). The synthetic route leading to the various trisubstituted pyrrole intermediates and products is presented in Scheme 1.

RESULTS AND DISCUSSION

As the primary objective was to study the feasibility of incorporating a methyl group into either the 3- or 4-position of a pyrrole ring *via* malonyl intermediates, no effort was made to develop synthetic methods to obtain regiospecific positional isomers. The synthesis of a regiospecific methyl substituted pyrrole fatty ester derivative was reported earlier by our group (4).

Treatment of methyl 10(11)-dicarbethoxymethyl-9,12-dioxooctadecanoate (**1a,1b**) with ammonium acetate yielded the pyrrole derivatives **2a,2b**, which contain a malonyl system at the 3- or 4-position of the pyrrole ring. The structure of the pyrrole system was characterized by the chemical shifts of the C-*H* and N-*H* protons of the *N*-heteroaromatic ring, which appeared at δ5.92 and 7.70, respectively.

Unlike the corresponding furan analogues, the ^{13}C nuclear magnetic resonance (NMR) chemical shifts of the ring C-2′ and C-5′ carbon nuclei of the pyrrole nucleus in compounds **2a,2b** were more shielded than those observed for the similarly positioned carbon nuclei in the furan. The shift of the C-2′ and C-5′ carbon atoms of the pyrrole ring appeared at the region of 127.9–130.8 ppm, while the shift of the C-2′ and C-5′ carbon atoms of the furan ring were found at 151.7–154.2 ppm.

Decarboxylation of one of the carboxylic acid groups in the malonyl moiety of compounds **2a,2b** was readily achieved when the mixture of compounds **2a,2b** was refluxed with sodium carbonate in methanol. Methylation of the decarboxylated product furnished compounds **3a,3b**. The presence of the CH_2COOCH_3 group in compounds **3a,3b** was confirmed by the singlet at δ3.38 for the shift of the methylene protons of the CH_2COOCH_3 substituent. When the hydrolyzed mixture of compounds **3a,3b** was heated at 180°C for 1 h, further decarboxylation took place to give the desired methyl substituted pyrrole derivatives (**4a,4b**). The methyl function attached to the pyrrole nucleus in compounds **4a,4b** was confirmed by the singlet at δ2.0 in the ^1H NMR spectrum and by the signal at 10.9 (pyrrole-CH_3) ppm in the ^{13}C NMR spectrum. The shift of the α-methylene carbon atoms located on either side of the pyrrole ring appeared at 25.8 and 27.7 ppm.

Pyrroles are generally more electrophilic than furans (5). This property of the *N*-heteroaromatic system appeared to accelerate the decarboxylation process during the conversion of the CH_2COOCH_3 substituent in compounds **3a,3b** to the CH_3 group in compounds **4a,4b**, which required heating at 180°C for only 1 h. In the case of the furan analogues of compounds **3a,3b**, conversion of the CH_2COOH to the CH_3 group required 4 h. From these results it can be concluded that the introduction of a methyl group into the ring of a pyrrole system *via* a malonyl group proceeds more readily than in the case of the corresponding furan analogue.

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Abbreviations: ECL, equivalent chain length; GLC, gas-liquid chromatography; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; R_f, retardation factor, TLC, thin-layer chromatography.

MATERIALS AND METHODS

Details of the chromatographic, analytical techniques and the method for the preparation of methyl 10(11)-dicarbethoxymethyl-9,12-dioxooctadecanoate (**1a,1b**) are described elsewhere (1).

*Synthesis of methyl 9,12-imino-10(11)-dicarbethoxymethyl-9,11-octadecadienoate (**2a,2b**).* A mixture of methyl 10(11)-dicarbethoxymethyl-9,12-dioxooctadecanoate (**1a,1b**, 1.0 g, 2.1 mmol), ammonium acetate (2.1 g, 27 mmol) and acetic acid (20 mL) was refluxed for 3 h. Water (50 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 50 mL). The ethereal extract was washed with water (20 mL) and dried over anhydrous sodium sulfate. The mixture was filtered and the solvent was evaporated. Silica gel column chromatographic separation of the residue using a mixture of light petroleum/diethyl ether (7:3, vol/vol) as the eluant gave a mixture of methyl 9,12-imino-10(11)-dicarbethoxymethyl-9,11-octadecadienoate (**2a,2b**, 0.88 g, 90%) as an oil. Retardation factor (R_f) (light petroleum/diethyl ether, 3:2, vol/vol) 0.3; infrared (IR) (cm^{-1}) (film) 3387(*w*), 1738 (*s*), 1033(*m*); ^1H NMR ($CDCl_3$,) 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.6 (*m*, 18H, C*H*$_2$), 2.31 (*t*, $J = 7$ Hz, 2H, 2-*H*), 2.52 (*t*, $J = 7.8$ Hz, 4H, C*H*$_2$-pyrrole), 3.66 (*s*, 3H, COOC*H*$_3$), 4.19 [*q*, $J = 7$ Hz, 4H, COOC*H*$_2$CH$_3$), 4.51 [*s*, 1H, C*H*(COOEt)$_2$], 5.92 (*s*, 1H, C*H* pyrrole) and 7.70 (*s*, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 13.9 (C-18), 14.1 (2C, COOCH$_2$*C*H$_3$), 22.6 (C-17), 24.9 (C-3), 25.8, 27.8, 29.0, 29.1, 30.1, 31.6 (C-16), 34.0 (C-2), 49.9 [*C*H(COOEt)$_2$], 51.4 (COO*C*H$_3$), 61.3 (2C, 2 × COO*C*H$_2$CH$_3$), 105.3 (c-4′ of pyrrole, assuming malonate ester substituent at C-3′ of pyrrole ring), 110.0 (C-3′ of pyrrole), 128.9 (C-2′ of pyrrole), 130.8, 131.0 (C-5′ of pyrrole), 169.5 (2C, 2 × *C*OOEt) and 174.0 (C-1) ppm.

*Decarboxylation of compounds **2a,2b**.* A mixture of compounds **2a,2b** (0.8 g, 1.7 mmol), methanol (20 mL), water (7 mL) and sodium carbonate (5 g) was refluxed for 12 h. The reaction mixture was acidified with dilute HCl (2M, 25 mL) and extracted with diethyl ether (2 × 40 mL). The ethereal extract was washed with water (20 mL) and dried over anhydrous sodium sulfate. The solvent was evaporated, and the residue was refluxed with borontrifluoride methanol complex (15%, w/w, 5 mL) and methanol (20 mL) for 15 min. Water (40 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 50 mL). The ethereal extract was washed with water and dried (Na_2SO_4). Silica gel column chromatographic separation of the residue gave compounds **3a,3b** (0.5 g, 78%) as an oil. R_f (light petroleum/diethyl ether, 3:2, vol/vol) 0.4; IR (cm^{-1}) (film) 3385(*w*), 1739(*s*), 1170(*m*), 1015(*m*); ^1H NMR ($CDCl_3$, δ) 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.8 (*m*, 18H, C*H*$_2$), 2.30 (*t*, $J = 7$ Hz, 2H, 2-*H*), 2.52 (*t*, $J = 7.8$ Hz, 4H, C*H*$_2$-pyrrole), 3.38 (*s*, 2H, pyrrole-C*H*$_2$COOCH$_3$), 3.66 (*s*, 6H, 2 × COOC*H*$_3$), 5.76 (*s*, 1H, C*H* pyrrole) and 7.64 (*s*, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 14.1 (C-18), 22.6 (C-17), 24.9 (C-3), 25.7, 27.7, 29.0, 29.2, 29.4, 29.5, 30.1, 31.7 (C-16), 32.1 (pyrrole-*C*H$_2$COOCH$_3$), 34.0 (C-2), 51.2 (COO*C*H$_3$), 51.8 (pyrrole-CH$_2$COO*C*H$_3$), 106.0 (C-4′ of pyrrole, assuming CH$_2$COOCH$_3$ substituent at C-3′ of pyrrole ring), 110.6 (C-3′ of pyrrole), 127.9, 128.1 (C-2′ of pyrrole), 130.6, 130.8 (C-5′ of pyrrole), 173.2 (pyrrole-CH$_2$-*C*OOCH$_3$) and 174.3 (C-1) ppm.

*Synthesis of methyl 9,12-imino-10(11)-methyl-9,11-octadecadienoate (**4a,4b**).* A mixture of compounds **3a,3b** (0.5 g, 1.3 mmol), potassium hydroxide (0.2 g) and aqueous ethanol (95%, 20 mL) was refluxed for 1 h. Dilute HCl (2M, 25 mL) was added, and the cooled reaction mixture was extracted with diethyl ether (2 × 20 mL). The ethereal extract was washed with water (20 mL) and dried (Na_2SO_4). The solvent was evaporated, and the residue was heated under nitrogen at 180°C for 1 h. The reaction product was refluxed with borontrifluoride methanol complex (15%, w/w, 5 mL) and methanol (20 mL) for 15 min. Water (30 mL) was added and the reaction mixture was extracted with diethyl ether (2 × 25 mL). Silica column chromatographic separation of the residue using a mixture of petroleum ether/diethyl ether, 7:3, vol/vol, as the eluant gave a mixture of compounds **4a,4b** (0.34 g, 82%) as an oil. R_f (light petroleum/diethyl ether, 3:2, vol/vol) 0.8; gas-liquid chromatography (SE-30) ECL = 20.4; ^1H NMR ($CDCl_3$, δ) 0.88 (*t*, 3H, C*H*$_3$), 1.2–1.8 (*m*, 18H, C*H*$_2$), 2.0 (*s*, 3H, pyrrole-C*H*$_3$), 2.30 (*t*, $J = 7$ Hz, 2H, 2-*H*), 2.47 (*t*, $J = 7.8$ Hz, 4H, pyrrole-C*H*$_2$), 3.66 (*s*, 3H, COOC*H*$_3$), 5.65 (*s*, 1H, C*H* pyrrole) and 7.3 (*s*, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 10.9 (pyrrole-CH3), 14.1 (C-18), 22.6 (C-17), 24.9 (C-3), 25.8, 27.7, 29.3, 29.7, 30.1, 31.7 (C-16), 34.1 (C-2), 51.4 (COO*C*H$_3$), 106.5 (C-4′ of pyrrole, assuming CH$_3$ substituent at C-3′ of pyrrole ring), 113.4 (C-3′ of pyrrole), 126.5, 126.7 (C-2′ of pyrrole), 130.1, 130.2 (C-5′ of pyrrole) and 174.3 (C-1) ppm.

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ARTICLE

Comparison of the Metabolism of α-Linolenic Acid and Its Δ6 Desaturation Product, Stearidonic Acid, in Cultured NIH-3T3 Cells

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The incorporation and metabolism of α-linolenic acid (18:3n-3) and its Δ6 desaturase product, stearidonic acid (18:4n-3), were compared by NIH-3T3 cells. In the presence of fetal calf serum, cells accumulated exogenously added 18:3n-3 and 18:4n-3 apparently at the expense of oleic acid (18:1n-9). Both 18:3n-3 and 18:4n-3 were elongated and desaturated to eicosatetraenoic acid (20:4n-3), eicosapentaenoic acid (20:5n-3) and docosapentaenoic acid (22:5n-3), but not to docosahexaenoic acid (22:6n-3), and were incorporated into phospholipids and triacylglycerols. Over a 4-d period, the growth of NIH-3T3 cells was slightly stimulated in the presence of 18:3n-3 (20 μg/mL) but was strongly inhibited in the presence of 18:4n-3 at the same concentration. This inhibition may be caused by enhanced lipid peroxidation as a result of the high levels of 18:4n-3 present. *Lipids 28,* **163-166 (1993).**

In recent years an increased dietary intake of fish oils, rich in long-chain n-3 fatty acids, has been vigorously promoted for its possible medical benefits. For instance, eicosapentaenoic acid (20:5n-3) may reduce the risk of thrombosis and, consequently, the risk of coronary disease (1,2). However, there is increasing evidence that excessive fish oil intake may induce adverse side effects (3,4). Some researchers have suggested that increasing the intake of α-linolenic acid (18:3n-3), the precursor of long-chain n-3 fatty acids, may provide the same beneficial effects but minimize the adverse effects of fish oil supplementation (5,6). Unfortunately, the activity of Δ6 desaturase in humans is relatively low (7), thus limiting the formation of long-chain n-3 fatty acids and, consequently, their potential beneficial effects.

Stearidonic acid (18:4n-3), the Δ6 desaturation product of 18:3n-3, is found in small quantities in borage and blackcurrant oils (8-10) and certain fish oils (for a review, see ref. 11). It has previously been shown in experimental animals that stearidonic acid is a more effective dietary supplement than α-linolenic acid in the formation of long-chain n-3 fatty acids (12). In other words, supplementing the diet with stearidonic acid could compensate for the low activity of Δ6 desaturase and low availability of its products.

To date few studies have compared the metabolism of stearidonic acid and α-linolenic acid in cultured cells. Previous studies in our laboratory have shown that administration of certain n-6 polyunsaturated fatty acids (PUFA) in the concentration range of 5-60 μg/mL kills many tumor cell lines in culture (13-15). We (16) and others (17) have also shown that 18:4n-3 was toxic to tumor cells in culture; however, its effect on a normal cell line has not been reported. In the present study, stearidonic acid and its direct precursor, α-linolenic acid, were added individually, to cell culture medium, and their effects on NIH-3T3 cells, a nonmalignant cell line, were compared. Cell growth was determined by cell counting, and the extent of incorporation and modification of 18:3n-3 and 18:4n-3 was determined in lipid extracts of the cells.

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Abbreviations: Chol, cholesterol; DMEM, Dulbecco's modified essential medium; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI + PS, phosphatidylinositol and phosphatidylserine; PUFA, polyunsaturated fatty acids; TG, triacylglycerol.

MATERIALS AND METHODS

Cell culture. NIH-3T3 cells, obtained from Dr. R. Bassin (NIH, Bethesda, MD), were maintained in Dulbecco's modified essential medium (DMEM) (Flow Labs, Mississauga, Ontario, Canada) containing 10% fetal calf serum (Flow Labs), without the addition of antibiotics. The protocol for the examination of the effects of 18:3n-3 and 18:4n-3 on cell growth has been described previously (18). Cells were seeded in 24-well plates at a density of 10^4 cells per well (0.5 mL) and allowed to attach. Twenty-four hours later (Day 0), the medium was replaced with fresh medium containing 5% fetal calf serum (total fatty acid content = 12.5 μg/mL medium; major fatty acids in percent: 16:0, 21; 16:1, 5; 18:0, 12; 18:1, 24; 18:2n-6, 6; 20:3n-6, 3; 20:4n-6, 11; 20:5n-3, 1; 22:4n-6, 1; 22:5n-6, 2; 22:5n-3, 3; 22:6n-3, 4) and either 18:3n-3 or 18:4n-3. Solutions of each fatty acid in ethanol (40 mg/mL) were diluted with medium to give final concentrations between 5 and 30 μg/mL. The final ethanol concentration was maintained at 0.2%. Control wells received fresh medium containing 0.2% ethanol. Cells were observed daily by phase contrast microscopy. After an additional 4 d in culture, cells were detached with trypsin and counted in a haemocytometer counting chamber. The trypan blue exclusion technique was used to determine the number of dead cells (19). Some experiments were carried out in the presence of ferrous ions ($FeCl_2.4H_2O$ 10 μg/mL) or dl-α-tocopherol (Sigma, St. Louis, MO; 10 μM).

Determination of fatty acid profiles. For lipid analysis, cells were seeded in 5 mL medium at a density of 10^5 cells per 6-cm Petri dish and supplemented with either 18:3n-3 or 18:4n-3 according to the protocol described above. After 3 d cells were released from the dish with trypsin, pelleted at 900 × *g* and resuspended in phosphate buffered saline (pH 7.4, composition as in ref. 20). For fatty acid quantitation, 17:0 was added to each sample as internal standard. Lipids were extracted by the method of Folch *et al.* (21), and the lipid class distribution was determined by high-performance liquid chromatography (HPLC) (22). Data were expressed as area percentage and were taken directly from the HPLC/mass detector analysis. Different lipid classes were expressed as a ratio which was calculated from the area percentage data for individual samples. For fatty acid analysis, phospholipids and triacylglycerols (TGs) were first separated by thin-layer chromatography (23). After transmethylation of lipid extracts (24), the fatty acid composition of the growth

medium, cells and phospholipid and TG fractions was determined by gas-liquid chromatography as described elsewhere (12).

All data are presented as mean ± SD (n = 4). Statistical analysis was performed using one-way analysis of variance and the Student's *t*-test using the SPSS-PC+ software package (SPSS Inc., Chicago, IL).

RESULTS

Effects of 18:3n-3 and 18:4n-3 on cell growth. Supplementation with either fatty acid (20 μg/mL) led to the appearance of lipid droplets within 24 h. The number of droplets decreased as time progressed. This observation has also been made when NIH-3T3 cells were incubated with other exogenous fatty acids (16:0, 18:2n-6, 18:3n-6 and 20:4n-6; Cantrill, Ells and Horrobin, unpublished data). Phase contrast microscopy revealed no other changes in cell morphology in either control or supplemented media. Cell number was not affected by either fatty acid during the first two days after supplementation. Thereafter, cells grown in 18:4n-3 supplemented medium ceased to proliferate, whereas cells grown in the control medium and those treated with 18:3n-3 continued to divide (Fig. 1). On Day 4 the number of cells in the 18:3n-3 supplemented medium was greater than in the control medium.

The effects of 18:3n-3 and 18:4n-3 supplementation at different concentrations are shown in Figure 2. On Day 4, 18:4n-3 at concentrations greater than 10 μg/mL had significantly suppressed the proliferation of NIH-3T3 cells and few viable cells remained at concentrations above 25 μg/mL. On the other hand, 18:3n-3 caused a 25–50% increase in cell number above control levels. The effect of 18:4n-3 was exacerbated by the addition of ferrous ions ($FeCl_2.4H_2O$ 10 μg/mL) and reduced by the simultaneous addition of dl-α-tocopherol (10 μM) (Fig. 2).

Cellular lipid distribution. To compare the effect of 18:3n-3 and 18:4n-3 supplementation on cellular lipid class distribution, cells were harvested, and analyses were performed on Day 3, before any cytotoxic effects were evident. Table 1 shows that the presence of either fatty acid caused a marked increase in the relative amount of TG and phosphatidylethanolamine (PE) and a decrease in the proportions of free cholesterol (chol) and phosphatidylcholine (PC) in total cellular lipids. The ratios of different lipid classes were calculated in order to determine if these relative changes were solely a reflection of TG formation or were more complex. Cells treated with 18:4n-3, as compared with 18:3n-3, had a 40% increase in the chol/PC ratio, a 20% increase in the PE/PC ratio and a 33% increase in the TG/chol ratio (Table 1). These results indicate that the two fatty acids have different effects on lipid class distribution.

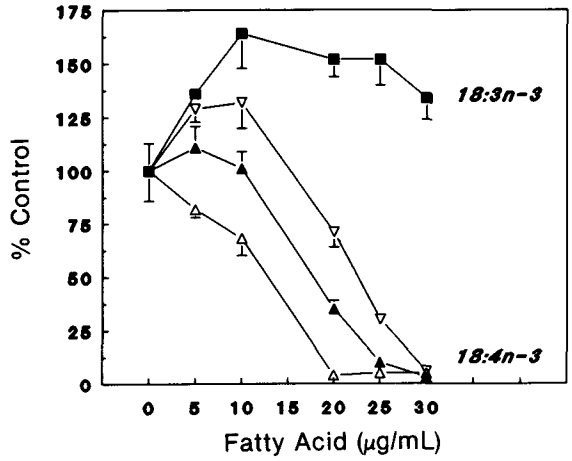

FIG. 2. Concentration-dependent effect of 18:3n-3 and 18:4n-3 on NIH-3T3 cells. Cells were cultured for four days in the presence of different concentrations of either 18:3n-3 (■) or 18:4n-3 (▲) between 0 and 30 μg/mL. 18:4n-3 treated cells were also grown in the presence of either ferrous ions ($FeCl_2.4H_2O$ 10 μg/mL) (△) or dl-α-tocopherol (10 μM) (▽). Data are presented as % control (total cell count at 0 μg/mL fatty acid) ± SD (n = 4).

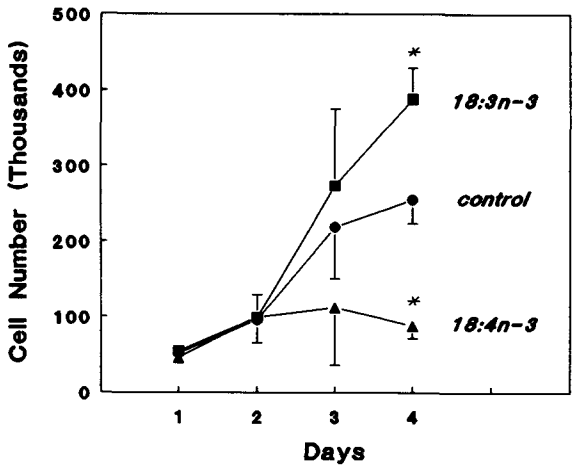

FIG. 1. Time-dependent effect of 18:3n-3 and 18:4n-3 on NIH-3T3 cells. Cells were cultured for four days in control medium (●), or in the presence of 20 μg/mL of either 18:3n-3 (■) or 18:4n-3 (▲). Data are presented as mean total cell count ± SD (n = 4). *Cell counts significantly different ($P < 0.05$) from control values on the same day.

TABLE 1

Lipid Class Distribution of NIH-3T3 Cells Treated for Three Days with 18:3n-3 or 18:4n-3[a]

	Control	18:3n-3	18:4n-3
Area %			
TG[b]	trace[c]	10.7 ± 1.0	15.9 ± 3.1[d]
Chol	14.5	10.5 ± 0.5	11.7 ± 1.0
PE	19.1	23.1 ± 0.9	22.2 ± 0.5
PI + PS	1.6	2.3 ± 0.1	0.9 ± 0.1[d]
PC	60.7	46.0 ± 0.6	36.8 ± 3.2[d]
SPM	trace	3.7 ± 0.2	3.6 ± 2.2
Ratio			
Chol/PC	0.24	0.23 ± 0.02	0.32 ± 0.04[d]
Chol/PE	0.76	0.45 ± 0.01	0.53 ± 0.04[d]
PE/PC	0.31	0.50 ± 0.04	0.60 ± 0.05
TG/Chol	—	1.03 ± 0.12	1.37 ± 0.35

[a]Results are expressed as area % ± SD (n = 4). For abbreviations, see footnote to title.

[b]May contain trace amounts of cholesterol esters.

[c]Present at less than 1%.

[d]Significantly different from 18:3n-3 treated cells ($P < 0.05$).

TABLE 2

Fatty Acid Composition of NIH-3T3 Cells Grown in Culture with Either 18:3n-3 or 18:4n-3 at a Concentration of 20 μg/mL[a]

Fatty acid	Day 1			Day 3		
	Control	18:3n-3	18:4n-3	Control	18:3n-3	18:4n-3
Total	32.2 ± 10.7	48.2 ± 14.7	32.2 ± 11.4	19.8 ± 2.3	30.8 ± 7.6	65.2 ± 9.7
18:3n-3	trace[b]	21.2 ± 6.4[c]	0.1 ± 0.0[d]	trace	5.2 ± 1.1[c]	0.3 ± 0.1[d]
18:4n-3	0.1 ± 0.1	1.5 ± 0.5[c]	10.7 ± 4.3[c,d]	0.2 ± 0.0	1.1 ± 0.2[c]	17.3 ± 2.1[c,d]
20:4n-3	0.1 ± 0.0	0.8 ± 0.3[c]	1.3 ± 0.5[c]	trace	1.2 ± 0.3[c]	6.7 ± 1.1[c,d]
20:5n-3	0.2 ± 0.1	1.2 ± 0.4[c]	1.3 ± 0.6[c]	0.1 ± 0.0	4.2 ± 1.0[c]	4.9 ± 1.1[c]
22:5n-3	1.3 ± 0.5	1.3 ± 0.4	0.9 ± 0.5	0.2 ± 0.0	1.4 ± 0.3[c]	1.4 ± 0.3[c]
22:6n-3	1.3 ± 0.5	1.1 ± 0.3	1.3 ± 0.9	0.3 ± 0.0	0.3 ± 0.1	1.5 ± 0.5[c,d]
Total n-3	3.1	27.1	15.6	0.8	13.5	32.3
% Total	9.5%	56%	48%	3.9%	44%	50%
Total n-6	7.0 ± 2.3	5.0 ± 1.6	3.1 ± 1.3	2.5	2.1 ± 0.5	5.2 ± 0.2
18:1n-9	7.2 ± 2.5	4.0 ± 1.2	3.5 ± 0.9	7.4	3.1 ± 0.7	6.5 ± 0.8

[a]Results are expressed as μg/10^6 cells, mean ± SD (n = 4).
[b]Indicates present in amounts below 0.1 μg/10^6 cells.
[c]Significantly different from control values ($P < 0.05$).
[d]Significantly different from 18:3n-3 treated values ($P < 0.05$).

Cellular fatty acid composition. Incubation with either 18:4n-3 or 18:3n-3 significantly increased the amount of n-3 fatty acids in total cellular lipid extracts. The n-3 fatty acids increased at the expense of monounsaturated, especially 18:1n-9, and n-6 fatty acids (Table 2). On Day 1 only modest amounts of elongation and desaturation products of either 18:3n-3 or 18:4n-3 were present, and no increases in the amounts of 22:5n-3 and 22:6n-3 were seen. By Day 3, the amount of the products was increased above Day 1 values. The major metabolite of 18:3n-3 was 20:5n-3, and the major metabolites of 18:4n-3 were 20:4n-3 and 20:5n-3. The amount of 22:6n-3 was increased above control values only in 18:4n-3 supplemented cells (Table 2).

The cellular fatty acid content (μg/10^6 cells) in 18:3n-3 treated cells was slightly higher than in control or in 18:4n-3 treated cells on Day 1 (Table 2). This resulted from an increase in total cellular n-3 fatty acids. In 18:3n-3 and 18:4n-3 treated cells, the levels of n-3 fatty acids were 27.1 and 15.6 μg/10^6 cells, respectively, and constituted approximately 50% of the total fatty acids. This was more than a fourfold increase above the levels present in control cells.

On Day 3, the total fatty acid content of both control and 18:3n-3 treated cells was reduced by 30% with respect to Day 1 values. However, the fatty acid content of 18:3n-3 treated cells remained 50% higher than that of control cells on Day 3. The fatty acid content of 18:4n-3 treated cells on Day 3 was twice that measured on Day 1. This value was also 2- and 3-fold higher than that of the 18:3n-3 treated and of the control cells, respectively, on Day 3. At this time, the levels of n-3 fatty acids were 4% of the total fatty acid content in control cells and 44 and 50% in 18:3n-3 and 18:4n-3 treated cells, respectively.

Table 3 shows the distribution of 18:3n-3 and 18:4n-3 and their metabolites in the TG and phospholipid fractions on Day 3. In the TG fraction, most of the n-3 fatty acids in the 18:3n-3 treated cells were recovered as 18:3n-3. However, a significant proportion of 18:4n-3 had been elongated to 20:4n-3 and subsequently desaturated to 20:5n-3 in the 18:4n-3 treated cells. In the phospholipid fraction, 20:5n-3 was the major product in both 18:3n-3 and 18:4n-3 treated cells, and a substantial amount of 20:4n-3 was also observed in the 18:4n-3 treated cells. The results also show that the amount of 22:5n-3 or of 22:6n-3 did not depend on the precursor fatty acid.

TABLE 3

Percent Distribution of n-3 Fatty Acids in Triacylglycerol and Phospholipid Fractions of NIH-3T3 Cells Incubated for Three Days with Either 18:3n-3 or 18:4n-3 (20 μg/mL)[a]

Fatty acid	Control	18:3n-3	18:4n-3
Triacylglycerols			
18:3n-3	0.2 ± 0.1	33.3 ± 1.3[c]	0.5 ± 0.1[d]
18:4n-3	0.5 ± 0.3	5.1 ± 0.5[c]	17.4 ± 6.3[c,d]
20:4n-3	trace[b]	7.6 ± 0.3[c]	27.9 ± 1.0[c,d]
20:5n-3	trace	8.0 ± 0.4[c]	13.6 ± 1.9[c,d]
22:5n-3	0.1 ± 0.1	3.8 ± 0.3[c]	4.1 ± 1.4[c]
22:6n-3	0.2 ± 0.1	1.1 ± 0.1[c]	0.8 ± 0.2[c]
Phospholipids			
18:3n-3	0.3 ± 0.1	22.4 ± 0.2[c]	1.3 ± 0.3[c,d]
18:4n-3	trace	3.2 ± 0.3[c]	9.9 ± 3.3[c,d]
20:4n-3	trace	3.1 ± 0.1[c]	10.7 ± 0.6[c,d]
20:5n-3	0.4 ± 0.1	11.9 ± 0.6[c]	15.2 ± 1.8[c,d]
22:5n-3	1.0 ± 0.1	3.0 ± 0.1[c]	4.0 ± 1.0[c]
22:6n-3	1.3 ± 0.2	1.1 ± 0.1	1.5 ± 0.2

[a]Results are expressed as mean ± SD (n = 4).
[b]Present at less than 0.1%.
[c]Significantly different from control values ($P < 0.05$).
[d]Significantly different from the 18:3n-3 values ($P < 0.05$).

DISCUSSION

Supplementation of growth medium with either 18:3n-3 or 18:4n-3 increased the quantities of n-3 fatty acids in NIH-3T3 cells. As shown in Table 2, however, the total fatty acid content on Day 3 was 30% lower than on Day 1 in both control and 18:3n-3 treated cells, whereas it was increased 2-fold in 18:4n-3 treated cells. The explanation for these differences is probably related to the rate of cell proliferation, since the number of 18:4n-3 treated cells had only doubled since Day 1, whereas the number of 18:3n-3 treated cells had increased 6-fold.

Treatment with 18:3n-3 increased the levels of the post Δ6 desaturase n-3 fatty acids (18:4n-3, 20:4n-3, 20:5n-3 and 22:5n-3) indicating that NIH-3T3 cells have an active Δ6 desaturase system (Table 2). In 18:4n-3 treated cells, there was an increase in 20:4n-3 levels but the level of 20:5n-3 was not affected. The data suggest that Δ5 desaturase activity was low in these cells and might have limited the production of 20:5n-3. Following either treatment, the amounts of 22:5n-3 and 22:6n-3 were similar on both Day 1 and Day 3.

It is also possible that the metabolism of n-3 fatty acids is not solely regulated by the activity of the desaturation and elongation enzymes but also by their incorporation into phospholipid and TG fractions. It has been proposed recently (25) that the distribution and metabolism of n-6 fatty acids is a consequence of the availability of acceptor molecules (PUFAs are predominantly found in the 2-position of phospholipids). There also exists the possibility of competition between n-3 fatty acids of different chain-length and degree of unsaturation for esterification into different positions of TG and phospholipid molcules.

An increased supply of n-3 fatty acids increased their incorporation into phospholipids and also led to the formation of TGs (Table 1). Acylation into TGs and the subsequent formation of intracellular perinuclear lipid droplets is evidence of another cellular response to excess PUFA intake. The formation of these lipid droplets was probably responsible for the changes in the lipid class ratios (Table 1). These changes could not be attributed solely to the increase in TG content since the proportions of different lipids were not reduced to the same extent. Thus, it is likely that the formation of the lipid droplet boundary membrane led to the alterations in the cholesterol/PC and the PE/PC ratios (Table 1). These shifts in the chol/phospholipid ratios may have also induced the incorporation of more PUFA into phospholipids.

If the incorporation of PUFA into phospholipid is restricted by the number of sites on suitable acceptor molecules, then excess fatty acid esterified into neutral lipids may be subsequently incorporated into phospholipid as cell number increases (26). In 18:3n-3 treated cells, the formation of long-chain metabolites and their accumulation in neutral lipid stores was lower than in 18:4n-3 treated cells (Table 3). This may have served to reduce the accumulation of substrates for lipid peroxidation since it has been shown previously that fatty acids with three and more double bonds are cytotoxic to many tumor cell lines in culture (14). This process is thought to be caused by the formation of lipid peroxides (27) since the cytotoxic effect of post Δ6 desaturase n-6 fatty acids cannot be prevented by certain cyclooxygenase or prostaglandin synthetase inhibitors (28). However, cell death may be accelerated by the addition of ferrous or copper ions and blocked by vitamin E and other antioxidants (18). Our observations support the involvement of lipid peroxidation in the lethal process (Fig. 2). The provision of 18:4n-3 to cells leads to the accumulation of substrates for lipid peroxidation, a process which is likely to cause cell death.

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The Effects of Dietary Fish Oil on Alveolar Type II Cell Fatty Acids and Lung Surfactant Phospholipids

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The purpose of this study was to determine the responsiveness of alveolar type II cells to dietary fish oil and the consequent effects on alveolar and lung surfactant. Rats were fed a corn oil or a fish oil diet for four weeks. Dietary n-3 fatty acids were readily incorporated into the type II cell phospholipids as indicated by higher levels of eicosapentaenoic acid (2.77 ± 0.10%) and docosahexaenoic acid (1.63 ± 0.10%) in the group receiving the fish oil diet. The elevated levels of n-3 fatty acids were accompanied by concomitant reduction in arachidonic acid and linoleic acid. Neither eicosapentaenoic acid nor docosahexaenoic acid was incorporated into type II cell triacylglycerols. Feeding a fish oil containing diet increased surfactant phospholipids, particularly 1,2-disaturated acyl phosphatidylcholines in whole lung compared to a corn oil diet. However, the amount of surfactant found in the alveolus was not different between the two diet treatment groups. The results suggest that dietary n-3 fatty acids stimulate synthesis and/or inhibit degradation of lung surfactant without altering surfactant secretion in alveoli.

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Surfactant is a lipoprotein complex that acts as a surface active agent to reduce the surface tension of the water lining the alveolar lumen for maintaining respiration (1). Also it plays an important role in the immune system by enhancing the activity of alveolar macrophages (2). Insufficiency of surfactant can cause respiratory distress syndrome in premature infants and adults (3,4). Surfactant is synthesized, stored and secreted by the alveolar type II cell, and all these processes regulate the availability of surfactant to alveoli (5,6). In an earlier study, we found that eicosapentaenoic acid stimulated surfactant secretion in cultured alveolar type II cells (Baybutt *et al.*, unpublished data). It is reasonable to assume that the stimulatory effect of eicosapentaenoic acid must be mediated first by its incorporation into tissue lipids.

The incorporation of dietary highly unsaturated long-chain fatty acids, eicosapentaenoic acid and docosahexaenoic acid has been demonstrated in humans (7,8) and animals (9-12). Specifically, these fatty acids are incorporated into various tissues such as kidney (11), liver (9-11,13) and brain (13) and different cell types such as macrophages (14), neutrophils (15), platelets (16) and erythrocytes (17,18). Little attention has been directed to the alveolar type II cell although some studies with lung tissue have been reported (9-11).

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Abbreviations: CL, cardiolipin; C-DMEM, complete Dulbecco's modified Eagle's medium; DSPC, 1,2-disaturated acyl phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; LPC, lysophosphatidylcholine; P, phosphorus; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; Sph, sphingomyelin; TLC, thin-layer chromatography; USPC, unsaturated acyl phosphatidylcholine.

The present study was conducted to determine whether alveolar type II cells can be enriched by eicosapentaenoic acid and docosahexaenoic acid by feeding rats a diet containing fish oil. In addition, the effect of dietary n-3 fatty acids on surfactant content and phospholipid composition in lung lavage and lung tissue was investigated. The results showed that surfactant phospholipid and 1,2-disaturated acyl phosphatidylcholine (DSPC) content increased in whole lung but not in the alveolus in response to fish oil feeding. The alteration in the amount of surfactant was accompanied by an increase of eicosapentaenoic acid in phospholipids of alveolar type II cells.

MATERIALS AND METHODS

Animals and diet. Pathogen-free male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Co. (Indianapolis, IN) and housed in stainless steel cages at approximately 24°C and 50% relative humidity with a cycle of 12 h light and 12 h dark (0600-1800 h). The rats, weighing 130-190 g, were fed *ad libitum* a semi-purified diet containing 15% corn oil or 13% menhaden oil plus 2% corn oil for four weeks. Water was provided at all times. The complete composition of the diets was adapted from those of others (19,20) and is listed in Table 1. The diet containing corn oil was rich in n-6 fatty acids (*e.g.*, linoleic acid) while the fish oil diet was high in n-3 fatty acids (*e.g*, eicosapentaenoic acid and docosahexaenoic acid) (Fig. 1). To minimize oxidation, the diet was packaged in small plastic ziplock bags, purged with nitrogen and stored frozen (−20°C). Food intake was determined by measuring the difference in the preweighed and unconsumed amount of diet. The unconsumed portion was discarded and replaced. Every other day, the food was administered and food intake and body weight were measured.

Animal care complied with guidelines developed by the Pennsylvania State University, the federal and state governments, and the National Research Council's Institute of Laboratory Animal Resources.

Isolation of type II pneumocytes. The type II pneumocytes were isolated according to the procedure of Mason *et al.* (21) with minor modifications (22). The procedure followed closely the original method (21) except that the carotid artery was cut instead of the aorta during the lung perfusion, and that percoll was used for the discontinuous gradient cell purification by centrifugation. The cells were resuspended in complete Dulbecco's modified Eagles medium (C-DMEM) salt supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) and immediately incubated under an atmosphere of 5% CO_2 and 95% air at 37°C. The nonadhering cells obtained after 3 h of incubation were further incubated for 20 h. The type II cells adhering to the culture plate, were washed to remove nontype II cells before being scraped and collected for lipid extraction. The purity of the adhering cells was determined microscopically after tannic acid staining (23).

Lung lavage and surfactant isolation. The lung lavage

TABLE 1

Diet Composition[a]

Ingredients	Fish oil diet	Corn oil diet
	(g/100 g diet)	
Casein	20.0	20.0
Dextrose	27.4	27.4
Corn starch	27.5	27.5
Menhaden oil	13.0	0.0
Corn oil	2.0	15.0
Cellulose (amorphous)	5.0	5.0
Salt/mineral mix (AIN-76A)	3.5	3.5
Vitamin mix (AIN-76)	1.0	1.0
DL-Methionine	0.3	0.3
Choline bitartrate	0.2	0.2
α-Tocopherol	0.075	0.075
t-Butylhydroquinone	0.003	0.003
Cholesterol[b]	0.079	0.079

[a]The diets were adapted from those used by other investigators (19,20).

[b]Cholesterol was added to the corn oil diet at an amount equivalent to that contained in fish oil.

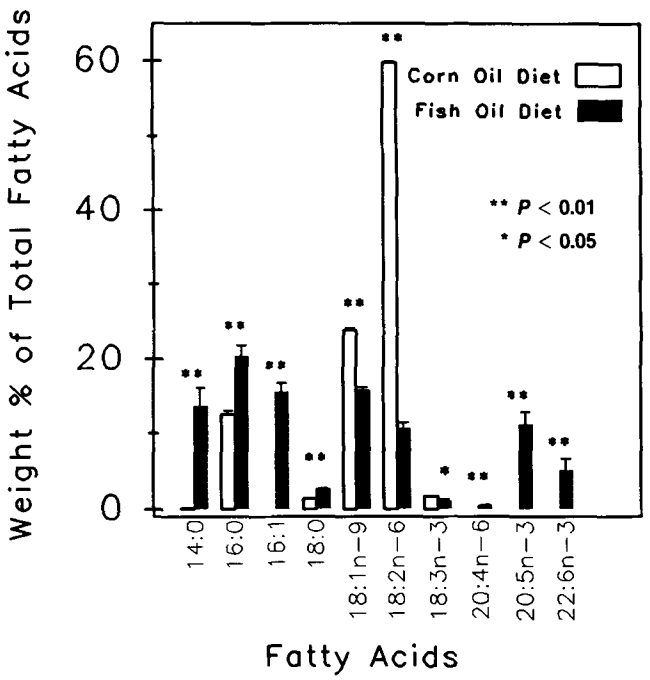

FIG. 1. The fatty acid compositon of the experimental diets is reported giving mean values with standard error for three samples. The wt% was calculated by dividing the weight of the fatty acid by the total fatty acid content and multiplying by 100. * and ** indicate significant difference between diets at $P < 0.05$ and $P < 0.01$, respectively.

was performed as previously described (24). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection. A cannula was placed in the trachea proceeding into the bronchi of the left lobe of the lung. The left lobe was chosen because it provided a manageable sample and was representative of whole lung (24). The lungs were removed from the animal, and the left lobe was instilled with approximately 6 mL of ice-cold 0.9% saline and lavaged. Three passes were made which has previously been shown to recover over 80% of the surfactant (24). The left lobe and whole lung of rats receiving similar dietary treatment were weighed for determining the weight proportion of the lavaged left lobe. This information was used to express the data relative to whole lung. The lavage fluid collected from three washes was pooled and centrifuged at 500 × *g* for 10 min to remove cells and cell debris. The supernatant was lyophilized and used for alveolar surfactant isolation. The lavaged lung (*i.e.*, left lobe) was dissected free of all connective and vascular tissue and homogenized in a medium consisting of 0.145 M NaCl in 0.01 M Tris buffer, pH 7.4, and 0.001 M ethylenediaminetetraacetic acid (EDTA), using a Ten Broeck homogenizer (Thomas Scientific, Philadelphia, PA) (25). The alveolar and tissue surfactants were isolated by the discontinuous sucrose gradient centrifugation procedure (26).

Lipid extraction and analysis. Alveolar type II cells, lung and lavage surfactant were extracted for lipids according to the method of Folch *et al.* (27). The extracted lipids were further separated into total phospholipid and triacylglycerol by thin-layer chromatography (TLC) using silica G plates, and the solvent system consisted of hexane/diethyl ether/acetic acid (80:20:1, by vol) (28). The phospholipids were visualized by exposing the plate to iodine vapor. The total phospholipid and triacylglycerol fatty acids of the type II cells were transmethylated with 14% boron trifluoride in methanol as described elsewhere (29). The methyl esters of fatty acids were analyzed by gas-light chromatography (Model 5880A, Hewlett-Packard, Palo Alto, CA) equipped with a hydrogen flame ionization detector using a glass column packed with 10% diethyleneglycol succinate + 1% phosphoric acid on 80/100 Chromosorb W AW (Supelco Co., Bellefonte, PA). The temperatures used were: injector, 200°C; column, 175°C; detector, 250°C. The flow rate of the carrier gas (helium) was 21.4 mL/min (30). Appropriate reference standards were used to determine retention times for identification of fatty acids. For quantification of the fatty acids, heptadecanoate was added as an internal standard at the methylation step (31). The mass was calculated as follows: fatty acid (wt) = {peak area of fatty acid/peak area of heptadecanoate} × weight of heptadecanoate added (32).

The individual phospholipids of lung tissue and lavage surfactant were separated by two-dimensional TLC on a Baker Si-250 silica gel plate (0.25 mm thick), with chloroform/methanol/petroleum ether (b.p. 60°C)/acetic acid/boric acid (40:20:30:10:1.8, vol/wt) as the developing solvent for the first dimension (33). Boric acid was dissolved in the methanol/acetic acid prior to combining with other solvents. The phospholipids were visualized by briefly exposing the plate to iodine vapor. Immediately after the removal of other phospholipids, phosphatidylcholine (PC) remaining on the plate was treated with 5% osmium tetroxide in carbon tetrachloride (33). The plate was developed in the second dimension for separation of DSPC using the developing solvent, chloroform/methanol/ammonium acetate (80:28:6, by vol). The individual phospholipids were quantified by determination of phosphorus content (34).

Data and statistical analysis. The phospholipid fatty acids of the alveolar type II cell are presented as weight

TABLE 2

Phospholipid and Triacylglycerol Fatty Acid Composition of Alveolar Type II Cells[a]

Fatty acid	Phospholipids		Triacylglycerols	
	Corn oil diet	Fish oil diet	Corn oil diet	Fish oil diet
	% of total fatty acids[b]			
14:0	4.3 ± 0.2**	7.3 ± 0.4	10.6 ± 1.7	13.5 ± 2.5
16:0	50.4 ± 0.3	51.4 ± 0.4	56.3 ± 3.0*	47.8 ± 3.3
16:1	9.9 ± 0.1**	13.2 ± 0.1	nd**	9.2 ± 2.8
18:0	6.7 ± 0.1**	5.8 ± 0.1	14.5 ± 2.0	15.7 ± 1.3
18:1n-9	9.0 ± 0.3	8.7 ± 0.1	11.5 ± 2.7	7.2 ± 0.6
18:2n-6	8.5 ± 0.2**	3.7 ± 0.3	4.7 ± 1.2	4.6 ± 0.5
18:3n-3	nd	nd	1.0 ± 0.5	0.9 ± 0.6
20:4n-6	8.2 ± 0.1**	3.2 ± 0.1	0.3 ± 0.3**	nd
20:5n-3	nd**	2.8 ± 0.1	nd	nd
22:4n-6	1.0 ± 0.0**	0.1 ± 0.0	nd	nd
22:5n-3	0.7 ± 0.0**	1.7 ± 0.1	nd	nd
22:6n-3	0.6 ± 0.0**	1.6 ± 0.1	nd	nd

[a]Mean values are reported with standard error for four samples. Each sample was pooled from four culture wells.

[b]The wt% was calculated by dividing the weight of the fatty acid by the total fatty acid content and multiplying by 100. Within the lipid class, * and ** signify differences between diets at $P < 0.05$ and $P < 0.01$, respectively. nd, Not detectable.

percent or μg fatty acid/10^6 cells. The amount of surfactant found in alveoli and lavaged lung is reported as nmoles phosphorus of phospholipids/lung, whereas the distribution of individual phospholipids in surfactant is expressed as molar percent of total phospholipids. Data are presented as mean ± standard error. Differences between the two diet groups were determined by a two-tailed *t*-test.

RESULTS

Effects of diets on body and tissue weights. The initial body weights were 162 ± 8 g and 160 ± 9 g for the fish oil fed and corn oil fed rats, respectively. As a result of similar food intake and body weight gain, there was no significant difference in their respective final body weights (295 ± 10 g *vs.* 292 ± 7 g). In addition, lung weights were the same between the fish oil fed and corn oil fed rats at the end of the study (1.26 ± 0.07 *vs.* 1.31 ± 0.06 g).

Fatty acids of alveolar type II cell. As determined by the tannic acid staining procedure, the purity of the alveolar type II cells was 86 ± 2%. Fatty acid profiles of phospholipids and triacylglycerol were analyzed. As shown in Table 2, phospholipids of alveolar type II cells derived from the fish oil fed group were enriched with eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) as compared to that of the corn oil group. The enrichment of eicosapentaenoic acid and docosahexaenoic acid was accompanied by reduction of arachidonic acid (20:4n-6), linoleic acid (18:2n-6) and stearic acid (18:0). Also, the contents of myristic acid (14:0) and palmitoleic acid (16:1n-7) in phospholipids were higher in the fish oil than corn oil fed groups. The dietary treatments had no effect on the content of palmitic acid, the predominant fatty acid of surfactant phospholipid.

The fatty acid distribution in triacylglycerol was essentially the same for both dietary groups (Table 2). However, palmitoleic acid was found only in the rats fed the fish oil diet. Fish oil feeding lowered palmitic acid content in triacylglycerols. Eicosapentaenoic acid and docosahexaenoic acid were not detected in triacylglycerol of the type II pneumocytes.

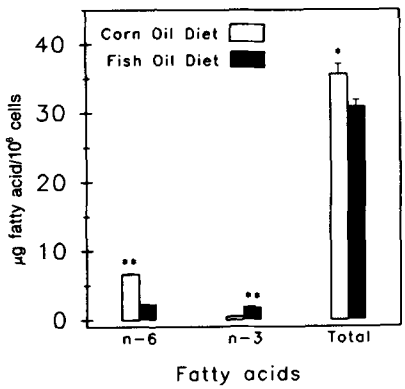

FIG. 2. The effects of dietary fats on n-6 and n-3 fatty acid mass of phospholipid in alveolar type II cells. Mean values are reported with standard error for four samples. Each sample was pooled from four culture wells. Data were derived from those shown in Table 2. * and ** indicate significant difference between diets at $P < 0.05$ and $P < 0.01$, respectively.

Consistent with the changes in wt% of phospholipid fatty acids, the mass of n-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) was higher and n-6 fatty acid (arachidonic acid and linoleic acid) was lower in fish oil than corn oil fed animals (Fig. 2). The mass of total fatty acids of phospholipid was slightly but significantly higher in the corn oil than the fish oil group. This finding was most likely the result of a greater reduction of long-chain n-6 fatty acids relative to the increase in n-3 fatty acids in response to fish oil feeding.

Lung tissue and lavage surfactant. The effects of dietary fats on surfactant contents in lung tissue and alveolar lavage were determined by measuring the amount of sur-

TABLE 3

Individual Phospholipid Content of Surfactant from Lavage and from Lung Tissue after Lavage[a]

PL[b]	Lung tissue		Lavage	
	Corn oil diet	Fish oil diet	Corn oil diet	Fish oil diet
	nmoles P/lung			
LPC	4.7 ± 1.2	6.4 ± 1.8	0.5 ± 0.3	2.5 ± 1.3
Sph	30.6 ± 2.2	30.5 ± 4.1	2.0 ± 1.2	2.0 ± 0.7
USPC	368.8 ± 41.5	463.2 ± 37.3	46.7 ± 10.7	45.7 ± 6.2
DSPC	620.6 ± 73.8*	860.1 ± 44.7	212.2 ± 14.5	227.7 ± 13.9
PI	27.9 ± 2.4**	46.8 ± 2.9	2.2 ± 1.1	3.6 ± 1.6
PS	33.1 ± 3.7**	49.7 ± 3.0	nd	nd
PE	114.4 ± 10.3*	182.4 ± 18.1	3.0 ± 1.3	5.9 ± 0.9
PG	76.7 ± 8.4	94.5 ± 6.0	14.0 ± 2.7	21.8 ± 1.8
PA	17.0 ± 1.9	22.2 ± 2.6	0.4 ± 0.3	0.3 ± 0.3
CL	7.1 ± 0.7	11.6 ± 4.9	0.6 ± 0.4	0.2 ± 0.2

[a]Mean values are reported with standard error for five samples.
[b]The individual phospholipids (PL) were separated by two-dimensional thin-layer chromatography (33). *and ** signify differences between diets at $P < 0.05$ and $P < 0.01$, respectively. nd, Not detectable. For abbreviations, see footnote to title.

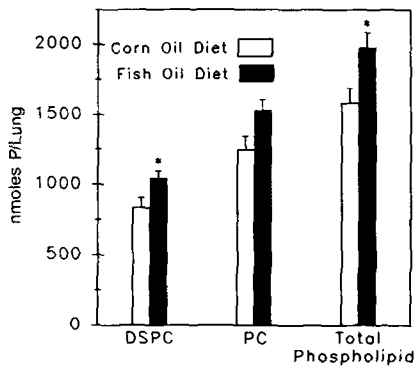

FIG. 3. The phospholipid content of whole lung surfactant. Mean values and standard error for five samples are presented. Data were obtained from Table 3 by combining the values for lavage surfactant and lavaged tissue surfactant. 1,2-Disaturated acyl phosphatidylcholine (DSPC) was separated from unsaturated acyl phosphatidylcholine (USPC) by two-dimensional thin-layer chromatography (33). Phosphatidylcholine was the sum of the DSPC and USPC. Total phospholipid was the sum of all individual phospholipid classes. *Indicates significant difference between diets at $P < 0.05$.

factant phospholipids. The amounts of lung tissue surfactant PC (1323 ± 75 nmoles P/lung) and total phospholipid (1768 ± 99 nmoles P/lung) measured in the fish oil fed animals were 34 and 36% higher than the corresponding values for the corn oil group. The mole% distribution of individual phospholipid was similar in the two dietary groups except for slightly higher phosphatidylinositol (PI) levels noted in the fish oil than in the corn oil group (data not shown).

However, when expressed as nmoles P/lung, there were apparent differences in the amounts of individual phospholipid classes resulting from the fats provided for the animals (Table 3). The fish oil feeding increased the amount of DSPC, PI, phosphatidylserine (PS) and phosphatidylethanolamine (PE) in lung tissue as compared to the corn oil feeding.

Unlike the alterations seen in lung tissue surfactant, the amounts of lavage surfactant PC (261 ± 18 nmoles P/lung) and total phospholipid (293 ± 17 nmoles P/lung) measured in the fish oil fed animals were not different from the corresponding values for the corn oil group. The contents of individual phospholipid classes in lavage surfactant were the same for both groups (Table 3).

When lung tissue and lavage surfactants were combined, the amount of DSPC (1046 ± 50 *vs.* 833 ± 73 nmols P/lung) and total phospholipid (1977 ± 109 *vs.* 1583 ± 110 nmoles P/lung) was again greater in the fish oil than in the corn oil fed animals (Fig. 3). This suggests an increase in total surfactant in the whole lung caused by fish oil.

DISCUSSION

Alveolar surfactant plays vital roles in pulmonary respiration and immune function. This together with the finding that eicosapentaenoic acid supplemented to tissue culture enhanced surfactant lipid secretion by alveolar type II cells prompted us to investigate the effect of dietary fish oil on surfactant availability in intact lung. In the present study, the amount of surfactant phospholipid (PL) was taken as a quantitative measure of surfactant because surfactant is composed of greater than 60% PL (35). As indicated by the increased amount of surfactant phospholipid in whole lung (*i.e.*, lavaged lung plus lavage surfactant), fish oil feeding can in fact augment the availability of surfactant. This finding suggests an overall increase in production and/or decrease in degradation of surfactant. It should be pointed out, however, that tissue associated surfactant is not as well characterized as alveolar surfactant and may not represent pure surfactant material (26). Thus the interpretation of the data must take into account these factors.

The reason for the observed increase of surfactant in

whole lung by fish oil is not known. Exogenous palmitic acid has been found to stimulate surfactant synthesis by alveolar type II cells (36,37). Also, maximal DSPC synthesis occurs when exogenous sources of palmitic acid are provided (38). This mechanism, however, may not explain the present observation since palmitic acid levels were either unaltered in phospholipids or decreased in triacylglycerol by fish oil feeding. Longmuir and Haynes (39) have suggested a cellular sorting mechanism favoring the incorporation of 14- and 16-carbon chain length fatty acids (including 16:1) into lamellar bodies in the alveolar type II cell. Therefore, the increased levels of 14:0 and 16:1n-7 in type II cell phospholipid and triacylglycerol of the fish oil fed rats may in part be responsible for the increased amount of surfactant in the lung.

In contrast to the stimulatory effect of eicosapentaenoic acid on surfactant secretion by cultured type II pneumocytes *in vitro* (Baybutt *et al.*, unpublished data), dietary fish oil rich in n-3 fatty acids did not alter the amount of surfactant found in the alveolus. However, this finding does not exclude the possibility that dietary n-3 fatty acid enhances surfactant secretion in intact lungs because the constant amount of surfactant found in alveoli could result from an increase in surfactant clearance.

In agreement with previous studies with whole lungs (10,11) and lung microsomes (9), the present study showed that dietary fish oil was effective in enriching eicosapentaenoic acid and docosahexaenoic acid in alveolar type II cell phospholipids. Not only did the relative proportion of eicosapentaenoic acid and docosahexaenoic acid to total fatty acids increase, the mass of these fatty acids was also higher in the fish oil fed than corn oil fed group. It should be noted that the increased n-3 fatty acid levels were accompanied by a reduction in arachidonic acid. There was little or no preformed arachidonic acid in the experimental diets containing either corn oil or fish oil (Fig. 1). Thus, the cellular content of arachidonic acid must be derived from its precursor, linoleic acid. It is then reasonable to speculate that the decreased content of arachidonic acid in type II cells of animals fed fish oil stems in part from the limited source of linoleic acid (Table 2) and from eicosapentaenoic acid inhibition on the conversion of dihomogamma linolenic acid to arachidonic acid (40). The low level of cellular linoleic acid in the fish oil fed group reflects a low content of the fatty acid in the diet (*i.e.*, 11%) as compared to 60% in the corn oil diet. A reduction of linoleic acid in whole lung has also been observed in rats fed a diet containing lard low in linoleic acid (10).

The failure to incorporate dietary eicosapentaenoic acid and docosahexaenoic acid into triacylglycerols of alveolar type II cell is in direct contrast to the significant accumulation of these long-chain n-3 fatty acids (9 and 11%, respectively) in liver triacylglycerols (12). Although the reason for the discrepancy observed between the type II cell and liver is not readily known, it may be related to the preferential incorporation of 14- and 16-carbon fatty acids into triacylglycerols *via* the lipid sorting mechanism within the alveolar type II cell (39), as mentioned above. The physiological significance of 14- and 16-carbon fatty acid enrichment in triacylglycerols is not known. It may be speculated that these fatty acids serve as a reserve for the synthesis of DSPC in the type II cells.

In summary, fish oil feeding modifies the lipid profile of the type II cell and increases whole lung surfactant.

ACKNOWLEDGMENTS

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Turnover of Cholesterol and Dipalmitoyl Phosphatidylcholine in Surfactant of Adult Rat Lung

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In order to compare the turnover of two major surfactant components, [1α,2α(n)-^{3}H]cholesterol and [*methyl*-^{14}*C choline*] dipalmitoyl phosphatidylcholine (DPPC) were introduced as lamellar bodies *via* the trachea into lightly anesthetized rats which were then allowed to recover. The radiotracers were assumed to have entered the alveolar surfactant pool and to have subsequently recycled in part into the lamellar bodies of alveolar type II cells. For DPPC, the specific activity *vs.* time curves of tubular myelin rich (alv-1) and tubular myelin poor (alv-2) alveolar lavage fractions were similar, and there was a plausible precursor-product relationship between lamellar bodies and either (or both) of these compartments. In contrast, however, the specific activities of alv-1 and alv-2 for cholesterol were quite different, allowing us to reject the hypothesis of a precursor-product relationship between classical lamellar bodies and alv-2. The estimated turnover time for DPPC in alv-1 was 240 or 206 min, depending on which subfraction of lamellar bodies one takes to be the precursor. For cholesterol it was 583 or 624 min. These longer turnover times for cholesterol should lead to a greater than twofold increase in the relative concentration of cholesterol in the putative product compartment. Such an increase was not found. We interpret this as reflecting either noncompartmental behavior of the alveolar surfactant pool, or multiple pools of lamellar bodies with different turnover times. We conclude that two major components of pulmonary surfactant, cholesterol and DPPC, are handled differently, and that for at least one of these substances, the widely accepted scenario of a compartmental precursor-product relationship between lamellar bodies and alveolar surfactant must be rejected.
***Lipids* 28, 173-179 (1993).**

The alveoli, and probably the distal airways, of the lungs would be mechanically unstable if the surface tension at the gas-liquid interface were the same as for saline or plasma. A mixture of phospholipids, neutral lipids and specific proteins, which can be recovered by lavage of lungs, is believed to supply the gas-liquid interface with a monolayer of surface-active material. The presence of this material, pulmonary surfactant, modifies the interface in at least two important ways: first, it reduces surface tension; second, it allows surface tension to vary between alveoli, permitting alveoli of different diameters to coexist.

The alveolar surfactant appears to be in a state of dynamic equilibrium, being replaced every few hours (1-3). This ongoing renewal raises a number of questions. Why is it necessary for there to be a turnover of surfactant? Could it not, like paint on a wall, be left to serve its purpose for many years? Does alveolar surfactant have a nominal life span analogous to the 120-d life of the red blood cell, or is it subject to a constant hazard such that its likelihood of being replaced is independent of the time already spent in the alveoli? Are the different components of surfactant replaced at different rates and, if so, does this reflect a multiplicity of turnover mechanisms?

Phospholipids make up about 77% of surfactant, with dipalmitoyl phosphatidylcholine (DPPC) being the most abundant. Phospholipids are found in alveolar surfactant in much the same proportion as found in the characteristic lamellar bodies of alveolar type II cells (4-6), and this has been taken as evidence that surfactant is synthesized in alveolar type II cells and stored and secreted as lamellar bodies. Electron microscopic evidence (7) suggests that lamellar bodies are precursors of alveolar surfactant, and this has been supported by radiotracer experiments (5).

If lamellar body DPPC is, indeed, the precursor for alveolar DPPC, then evidence from several laboratories points to there being substantial recycling of this component of surfactant (3,8). This argues that the impetus for surfactant turnover is not the destruction of individual molecules, for they could not then be recycled without modification. Several alternative reasons for recycling might be proposed, but it suffices to divide these into two broad categories: those which would necessarily involve all components (phospholipid, neutral lipid, protein) in the same turnover kinetics, and those in which similar kinetics would only occur as a matter of unlikely coincidence.

Tracer experiments depend in part on the fact that if two distinct molecular species enter a pool and exist within the pool in the same relative concentrations then they have the same residence time there. The early observation that lamellar bodies and alveolar surfactant yield the same relative concentrations of different phospholipids implied a similar turnover and was the early justification for studying phospholipid turnover as a whole without distinguishing between individual phospholipids (4). Individual phospholipids, however, have different physical and biochemical properties, and it would be a remarkable coincidence if, despite these differences, the independent and individual removal of phospholipid molecules were to result in identical mean residence times in the alveoli for distinct molecular species. If, however, the removal of molecules does not represent independent events, then similar turnover times might be not only possible but actually obligatory. If, for instance, local alveolar collapse were to lead to surfactant bilayer or vesicle formation and if the involved surfactant were then sufficiently stable that the original monolayer configuration could not be restored, then all components in the affected area might be destined for recycling. Baritussio *et al.* (1) note the logical nexus between bulk uptake processes and a lack of molecular specificity, and have demonstrated a remarkable constancy in phospholipid concentration

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Abbreviations: alv-1, tubular myelin rich alveolar lavage fraction; alv-2, tubular myelin poor alveolar lavage fraction; DPPC, dipalmitoyl phosphatidylcholine; lbA, lamellar bodies, classical; lbB, lamellar bodies, vesicular.

between nine phospholipids in two subfractions of alveolar lavage.

The similarity of residence times for different phospholipids suggested that bulk uptake might be a quantitatively dominant event in precipitating surfactant turnover. Not only would it dictate similar turnover times, but it would justify there being turnover when individual molecules, taken in isolation, were unchanged and seemingly in no need of being removed. Indeed, it would justify not only removal, but also the recycling of the individual molecules once repackaged into a physiologically useful configuration. Lamellar bodies once released into the alveolar hypophase release their contents in a morphologically distinct form called tubular myelin which may be the precursor of the more amorphous surfactant to be found at the gas-liquid interface. A plausible relationship between these pools is shown in Figure 1.

The distinct and structured architecture of tubular myelin lends support to the view that functioning surfactant depends on the well-ordered arrangement of the component molecules, and that a disturbance of this arrangement might necessitate recycling in order to restore a functioning architecture.

Appealing though such a conjecture might be for events precipitating surfactant removal, the evidence is based on the implied similar turnover times for relatively similar phospholipid molecules. We felt that the conjecture should be tested using dissimilar components such as cholesterol and DPPC which make up approximately 10 and 50%, respectively, of surfactant.

In these experiments we therefore introduced tracer amounts of radiolabelled DPPC and cholesterol *via* the trachea into the lungs of lightly anesthetized rats which were then allowed to recover. The tracers were introduced as lamellar bodies, the limiting membranes of which had been ruptured immediately prior to instillation. The disappearance of these tracers from the alveolar compartment of the recipient rats and the partial recycling of the tracers into the lamellar bodies of those rats were followed over the next 24 h. From these data the turnover times of the tracers in the alveolar compartment can be deduced and compared, as can the plausibility of a precursor-product relationship between lamellar bodies and the alveolar compartment. This experimental protocol, including the preliminary preparaton of radiolabelled lamellar bodies, is outlined in Figure 2.

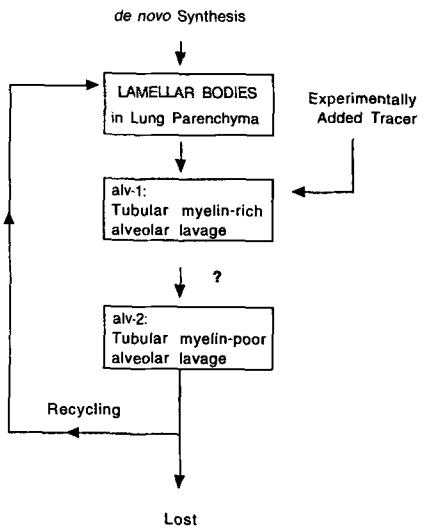

FIG. 1. The flow between proposed compartments may involve both *de novo* synthesis and recycling of surfactant components which are stored in lamellar bodies. These are released, or experimentally introduced, into the alveolar hypophase where we identify it as a tubular myelin-rich pool (alv-1). This may feed into a tubular myelin-poor pool (alv-2) before being either recycled or lost from the system.

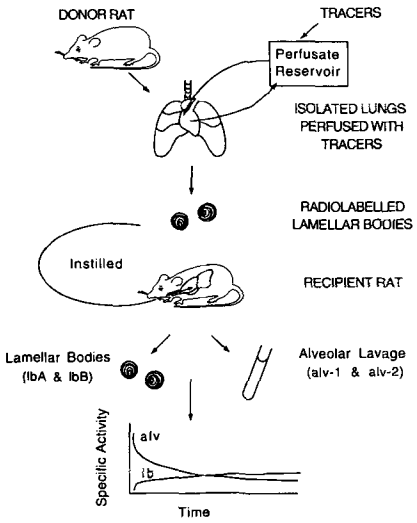

FIG. 2. The experimental protocol involves using an isolated perfused rat lung to prepare radiolabelled lamellar bodies (lbA and lbB). These are introduced *via* the trachea into the lungs of recipient rats and are assumed initially to enter the tubular myelin rich alevolar lavage fraction (alv-1) pool. The movement of the tracer molecules into other pools is followed over time, and from the specific activities of these pools, putative precursor-product relationships and turnover times can be inferred.

MATERIALS AND METHODS

All of the experiments were performed on specific pathogen-free male Porton rats (250–290 g) which were maintained in a positive pressure filtered environment and fed normal rat pellets (Milling Industries, Adelaide, Australia) and water *ad libitum.*

Preparation of radiolabeled lamellar bodies. Labeled lamellar bodies were prepared by reperfusing a reservoir containing radiolabeled precursor molecules through an isolated rat lung (3) which was set up as previously described (9). Briefly, the rat was heavily anesthetized with methohexital sodium (Eli Lilly, Sydney, Australia; 160 mg/kg i.p.) and ventilated with 5% CO_2/95% O_2 at 60 breathes/min, a tidal volume of 2.5 mL and end-expired pressure of 2 cm H_2O. The thorax was opened, and catheters were placed in the main pulmonary artery *via* the right ventricle and in the left atrium *via* the left ventricle. Without interruption of the circulation, the lungs were perfused at 10 mL/min with Krebs bicarbonate solution containing 4.5% bovine serum albumin (Cohn fraction V, Sigma, St. Louis, MO). Finally, the lungs were removed from the thorax and placed in a closed chamber saturated with water vapor at 37°C; the positive pressure ventilation continued. The perfusate was recirculated through a 25-mL reservoir which contained 100 μCi

[1α,2α(n)-^{3}H]cholesterol (46.2 Ci/mmol, Amersham, Sydney, Australia) and 10 μCi of [*methyl*-^{14}C]choline chloride (56.0 mCi/mmol, New England Nuclear, Boston, MA). The radiolabeled cholesterol in toluene was evaporated to dryness and then 25 mL of Krebs bicarbonate/albumin added. This was then warmed to 37°C, sonicated and stirred vigorously before the radiolabeled choline chloride was added. After 2 h of perfusion, the vascular bed was flushed free of radiolabel with 50 mL of unlabeled perfusate that was not recirculated.

Preparation of lamellar body fraction. We used a modification of the standard Duck-Chong method (10) and further fractionated this as previously described (11) to prepare fractions of greater uniformity. Briefly, the lungs were minced and then homogenized in 2 mL of 1.0 M sucrose. The homogenate was centrifuged at 1,000 × *g* (avg) for 5 min at 2°C, and the supernatant was aspirated and adjusted to 1.3 M with 2 M sucrose. Sucrose gradients were layered on the homogenate in 1 mL vol, each of 0.1 M, from 0.8 to 0.3 M (0.4 and 0.5 M were 0.5 mL vol) and the tubes were centrifuged at 80,000 × *g* (avg) for 2 h at 2°C. The band corresponding to lamellar bodies (between 0.4 and 0.5 M) was removed and diluted with an equal volume of cold distilled water, mixed gently, then centrifuged to prepare subfractions; lbA 8,000 × *g* (avg) for 30 min at 2°C; and lbB, 80,000 × *g* (avg) for 60 min at 2°C. Only the subfraction containing classical lamellar bodies as determined by transmission electron microscopy, and which we denote lbA, was used for tracheal instillation in the studies reported here.

Method of lung lavage. The lungs were degassed at 0.5 atm for 60 s and then lavaged at 2°C with three separate 10-mL vol (for a 250-g rat) of 0.15 M saline, each volume instilled and withdrawn three times (4). This recovered 73% of the phospholipids that could be recovered from ten triple lavages.

Analysis of phospholipids. Lipids were extracted in chloroform/methanol/water (1:2:0.8, by vol), the disaturated phospholipids were separated by the osmium tetroxide method, and the phospholipid content was determined by measuring inorganic phosphorus as described previously (4).

Analysis of cholesterol. Cholesterol was determined using high-performance liquid chromatography as previously described (12). Briefly, separation used prepacked μPorasil columns (15 cm × 3.9 mm) from Waters Associates (Milford, MA), a mobile phase consisting of hexane/isopropyl alcohol/acetic acid (750:10:0.1, by vol) and a variable wavelength detector (model 481, Waters Associates) at 206 nm.

Intratracheal instillation of lamellar bodies. The lbA fraction was instilled as previously described (3). Briefly, the limiting membranes were ruptured by incubation for 5 min at 37°C, and two separate 75-μL aliquots were rapidly instilled through a fine catheter inserted between cartilage rings of the trachea and advanced to the level of the carina, one into the left, and one into the right, bronchus. The amount of DPPC instilled was approximately 17 μg, containing 24,000 dpm, and the amount of cholesterol instilled was approximately 3 μg, containing 20,000 dpm per rat.

Statistical methods. Turnover time in the product compartment was estimated by a direct least squares fit to the untransformed data as outlined in the Appendix. We have previously shown (13) that this approach gives unbiased estimates of turnover time. Because the analysis depends on a comparison of the turnover times of two compounds, data was only included from experimental animals in which both compounds had been successfully assayed. The hypothesis of steady state compartmental precursor-product relationship may be tested using a test statistic analogous to a variance ratio (14). Because of the nonlinearity of the model and because the data from different times have different variances, the test statistic will vary from an F distribution. The distribution of the test statistic was therefore derived using a Monte-Carlo approach. Random number generation within the Monte-Carlo trials was carried out by applying a randomizing shuffle (15) to the sequence of random numbers generated by the Turbo Pascal subroutine *Random*.

RESULTS

The amount of DPPC and cholesterol in the tissue fractions lbA and lbB, and in the alveolar compartment fractions alv-1 and alv-2 are listed in Tables 1 and 2.

The ratios of cholesterol to DPPC (wt/wt) in lbA (0.077), lbB (0.17), alv-1 (0.11) and alv-2 (0.18) confirm that the two lamellar body compartments are biochemically dissimilar, as are the two alveolar subfractions. The rapid fall in

TABLE 1

Pool Sizes and Specific Activities for DPPC[a]

	Pool and size μg/g dry lung			
	lbA	lbB	alv-1	alv-2
Size	801 ± 82	554 ± 62	856 ± 109	2899 ± 122
Time (h)	Specific activity (dpm/μg phosphorus)			
0.5	110 ± 21.3 (7)	69 ± 12.7 (6)	372 ± 53.2 (6)	425 ± 76.9 (7)
1	143 ± 31.7 (11)	75 ± 6.1 (9)	383 ± 47.3 (10)	337 ± 31.3 (11)
2	117 ± 11.9 (18)	107 ± 11.0 (17)	340 ± 31.4 (17)	330 ± 22.5 (18)
4	84 ± 11.1 (4)	71 ± 7.9 (6)	199 ± 17.0 (6)	189 ± 13.1 (6)
8	69 ± 7.1 (5)	53 ± 4.0 (6)	131 ± 14.2 (6)	130 ± 11.6 (6)
24	19 ± 3.1 (6)	18 ± 2.4 (6)	40 ± 4.5 (6)	32 ± 3.7 (6)

[a]Results are expressed as mean ± SEM, with number of rats in parentheses. Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; lbA, lamellar bodies (classical); lbB, lamellar bodies (vesicular); alv-1, tubular myelin rich alveolar lavage fraction; alv-2, tubular myelin poor alveolar lavage fraction.

TABLE 2

Pool Sizes and Specific Activities for Cholesterol[a]

	Pool and size μg/g dry lung			
	lbA	lbB	alv-1	alv-2
Size	62 ± 6.3	96 ± 8.6	91 ± 8.0	519 ± 25
Time (h)	Specific activity (dpm/μg cholesterol)			
0.5	91 ± 22.0	64 ± 9.3	512 ± 69.2	296 ± 29.6
1	96 ± 21.4	42 ± 7.7	417 ± 64.3	257 ± 12.1
2	139 ± 31.3	64 ± 9.5	301 ± 33.1	159 ± 18.3
4	63 ± 13.9	80 ± 25.5	277 ± 42.2	138 ± 16.7
8	53 ± 11.4	43 ± 5.6	230 ± 40.0	102 ± 7.6
24	20 ± 3.8	16 ± 3.6	69 ± 5.5	23 ± 2.8

[a]Results are expressed as mean ± SEM. Rat numbers and abbreviations are identical to those in Table 1.

specific activity of both DPPC and cholesterol in the alveolar compartment was coincident with a rapid initial increase in specific activity of these two lipids in both of the lamellar body fractions, suggesting that there is considerable recycling of lipid from alveolus to lamellar bodies.

An example of the analysis of possible precursor-product relationships between these four fractions is illustrated in Figure 3, which addresses the relationship between the classical lamellar body compartment lbA and the tubular myelin rich alveolar subfraction alv-1, with DPPC as the tracer molecule. For DPPC it is widely held that lbA is the precursor of alv-1, that both pools act as homogeneous well mixed compartments and that both the turnover time of DPPC in alv-1 and that the extent of recycling into lbA can be calculated on the basis of those assumptions. Figure 3 illustrates the analysis of the experimental data on the basis of those assumptions. The observed decline

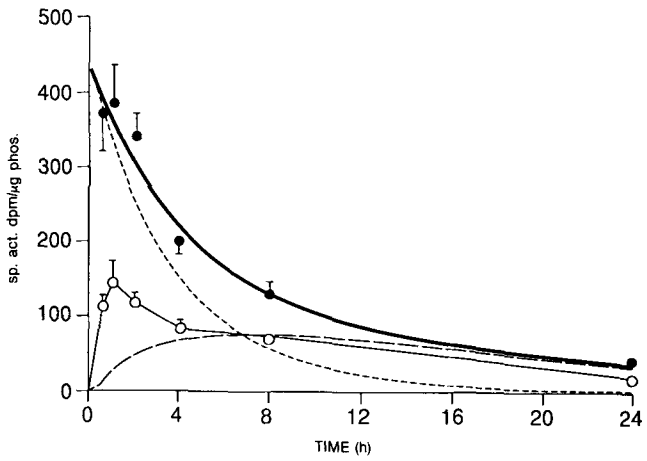

FIG. 3. Specific activities of dipalmitoyl phosphatidylcholine, measured as dpm per μg of phosphorus (sp. act. dpm/μg phos.) in the classical lamellar bodies from lung parenchyma (○), and in the tubular myelin-rich alveolar lavage (●) following intratracheal introduction of tracer is consistent with a turnover time of 240 min. The curve of best fit (weighted least squares) for the alveolar compartment (▬) is the sum of an exponential decay with 240 min time constant (- - -), and recycling from the newly synthesized lamellar bodies (— —) with the same time constant. Least squares estimates of the lamellar body specific activity is linearly interpolated (—) between sampling times.

TABLE 3

Analysis of Precursor-Product Relationships[a]

Tracer	Precursor	Product	Prob.	Turnover (min)	Recycle (%)
DPPC	lbA	alv-1	0.26	240 ± 38	46
	lbA	alv-2	0.29	206 ± 31	49
	lbB	alv-1	0.13	300 ± 47	36
	lbB	alv-2	0.17	254 ± 37	39
Cholesterol	lbA	alv-1	0.30	583 ± 55	24
	lbA	alv-2	0.02	180 ± 39	56
	lbB	alv-1	0.32	624 ± 52	18
	lbB	alv-2	0.14	198 ± 41	47

[a]The probability (Prob.) is the proportion of Monte-Carlo trials in which the trial test statistic exceeded that calculated from the real experiment. The turnover time is in minutes ± SD. For each precursor-product pair, 1000 simulations were carried out. The recycle % is the percentage of the product total radioactivity which can be attributed to recycling from lamellar bodies within the 24-h duration of the experiment. Abbreviations found in Table 1.

of the specific activity of DPPC in alv-1 represents the sum of two components. One component is an exponential decay, with time constant $\tau = 240$ min, of the initially introduced dose. This first component dominates the initial response. The second component represents recycled DPPC and is influenced by the appearance of tracer in the lbA compartment and the subsequent secretion of these labelled lamellar bodies into the alveolar compartment. This second component of alv-1 specific activity follows the lbA specific activity, approaching it always with the same time constant $\tau = 240$ min. Although its contribution is initially small, this second component dominates the latter part of the experiment.

Table 3 summarizes the result of such an analysis for both DPPC and cholesterol, taking either lbA or lbB as precursor and alv-1 or alv-2 as product.

DISCUSSION

The results for DPPC are broadly in line with the expectations. The widely accepted precursor-product relationship between lbA as precursor and alv-1 as product is not rejected by our statistical test, and the least-squares estimate of turnover time is close to our previous estimates (3). The specific activities in alv-1 and alv-2 are very similar. Our analysis accepts either lbA or lbB as precursor, and either alv-1 or alv-2 as product, deriving in each case similar time constants.

In constrast, the specific activities of alv-1 and alv-2 for cholesterol are quite different, suggesting that the alveolar kinetics of cholesterol differ from those of DPPC. The statistical analysis reflects this difference in specific activity and rejects the hypothesis that alv-2 is derived from lbA as sole precursor. There is a plausible precursor-product relationship ($P = 0.14$) between lbB and alv-2. The product alv-1 could be derived either from lbA or lbB as precursor. The time constants associated with these scenarios are, however, very much greater for cholesterol than for DPPC.

Were we to accept lbA as precursor, and alv-1 as product, then the 583 min time constant for cholesterol turnover in alv-1, taken in conjunction with the 240 min time constant for DPPC in alv-1, would lead us to expect that

the ratio of cholesterol to DPPC in alv-1 should be 583/240 = 2.4 times greater than the ratio of the two in lbA. This factor of 2.4 derives from the proposition that on average, cholesterol molecules reside in the alv-1 compartment 2.4 times longer than do DPPC molecules. The relative increase observed, however, was only 23%, and this is inconsistent with their having a common origin in lbA and the turnover times as calculated.

The alternative scenario of lbB as precursor and alv-1 as product is even less plausible. Not only does it involve a sequence of events which differs from accepted opinion, but it predicts by an argument analogous to that above, that in going from lbB to alv-1 there should be a relative *increase* in the cholesterol to DPPC ratio of more than twofold. Instead we note an observed *decrease* of 39%.

The precursor-product relationship which is plausible (although barely so) for both DPPC and for cholesterol, and for which the observed cholesterol/DPPC ratios are not in strong conflict with those predicted from the turnover times, is for lbB as precursor and alv-2 as product. Here, there is a decrease in cholesterol/DPPC ratio of 23% predicted from the turnover times against an observed increase of 3%. Perhaps the best one can say here is that the grounds for rejecting this scenario are not as strong as for the others discussed. Against this scenario, we note that lbB is the vesicular fraction and lacks the lamellae which are widely presumed to be 'packaged' surfactant. Further, alv-2 specifically lacks the tubular myelin which electron microscopic evidence argues to be the form taken by surfactant newly secreted from the alveolar type II cell.

The data therefore lead us to two tentative conclusions. First, that the apparent kinetics of cholesterol in lamellar bodies and alveolar surfactant under the experimental conditions described for the rats in this experiment, differ markedly from those of DPPC. In particular, if lbA is the sole precursor of alv-1 for DPPC, then this relationship cannot hold for cholesterol, and *vice versa*. Second, that if despite this evidence we persist in postulating that lbA is the common precursor for alveolar cholesterol and alveolar DPPC, then the differing turnover times precludes bulk uptake as the dominant mechanism of surfactant removal from the alveoli.

We can only speculate on the mechanism underlying these results. It is useful to consider our results in conjunction with those of Pettenazzo *et al.* (16) who have reported the results of similar experiments in the adult rabbit. In those experiments, the form in which the surfactant lipids were introduced had a profound effect on the turnover kinetics. When instilled as liposomes *via* the trachea, phosphatidylcholine and cholesterol had identical kinetics of removal from the alveolar compartment. However, the kinetics of liposome removal differed from those of a saline-suspended tubular-myelin-rich fraction, but again, for material instilled down the trachea as a tubular-myelin-rich fraction, the kinetics for phosphatidylcholine mirrored those for cholesterol. In the experiments of Petenazzo *et al.* (16), therefore, the form in which the tracer was instilled was pivotal, but the removal from alveoli did not distinguish between cholesterol and phosphatidylcholine.

In the present experiments, a clear difference exists between the behavior of cholesterol and that of DPPC in the rat. Although the difference may reflect the species, a more interesting possibility revolves around our introduction of the tracers within lamellar bodies. Removal of material from alveoli might well depend on whether it is recognized as a foreign body, as nonfunctioning surfactant or as physiologically functioning surfactant. We have introduced the surfactant in the form of lamellar bodies in the expectation that much of it will be treated as functionally normal surfactant. Consistent with this, we would interpret our results as suggesting that, in functionally normal rat surfactant, cholesterol and DPPC exhibit different kinetics of removal from the alveolar pool. We speculate that when these tracers are introduced as liposomes or as resuspended, processed lavage subfractions a significant proportion is treated either as foreign body or as nonfunctioning surfactant, and as such is subject to removal mechanisms which might not be expected to distinguish between the tracer molecules.

If we accept that the two tracers, when introduced as lamellar bodies intratracheally in the rat, differ in their kinetics, the question arises as to why cholesterol kinetics should differ from those of DPPC. Three interpretations suggest themselves.

First, cholesterol introduced as lamellar bodies *via* the trachea might not be acting as a tracer, and the aberrant behavior of alveolar cholesterol might be an artifact of the experiment. Against this, we note that DPPC introduced in the same lamellar bodies has behaved as expected and therefore presumably has acted as a tracer. Within the macrophages, the time course of the specific activity for DPPC and for cholesterol do not differ greatly. It is difficult to see why one substance would act as a tracer while another, introduced in the same lamellar body preparation, would not, but the possibility exists that DPPC might have dispersed from a local aggregation of introduced surfactant leaving a relatively immobile glob enriched in cholesterol.

A second possibility is that the introduced cholesterol acts as a tracer but that the alveolar pool is not a homogeneous compartment with respect to cholesterol. The experimental data is consistent with alveolar cholesterol existing as at least two compartments, one of which has a slow turnover. As a variation on this theme, alveolar cholesterol might be in rapid equilibrium with a poorly lavageable cholesterol compartment from which DPPC is excluded. While these scenarios are compatible with our results, we would not necessarily advocate them as the most likely explanations.

A third possibility is that alveolar cholesterol represents a single homogeneous compartment and that the apparent discrepancy in the results reflects recycling of cholesterol from a pool of higher specific activity than the lamellar bodies as analyzed. Either this is an entirely different source or the lamellar bodies themselves exist as distinct subpopulations of different composition, specific activity and turnover kinetics, as suggested by Young and Tierney (17). There is some support for this speculation in recent work from our laboratory concerning the effect of breathing pattern on alveolar and tissue surfactant (18).

Our working hypothesis is that the optimal physical characteristics of surfactant depend on the pattern of breathing. Reflecting this, the surfactant composition which is optimal in the resting individual might differ from that required during exercise. It is conceivable that the lipid composition of surfactant might change with activity in the mammal as it does with temperature in the

lizard *Ctenophorus nuchalis* (19). In fact, we have recently found that the ratio of cholesterol to DPPC in the alveolar compartment varies with exercise in both rats and humans, and this appears to reflect a differential release of these two components (20,21). The constant turnover of surfactant would then be a necessary consequence of matching surfactant composition to the activity of the mammal at the time. Changes on a short time scale might be accommodated by drawing on pools of lamellar bodies of different composition, and our results are consistent with there being within the surfactant precursor pool a subset of precursors with more rapid recycling. Such a subset would attain a higher than average specific activity following the tracheal instillation of tracer. The data is interpretable as evidence for the feeding of alveolar lavage cholesterol preferentially from a subset of precursors rich in cholesterol and with a rapid turnover.

APPENDIX

Turnover of surfactant components is difficult to quantify, even when seemingly drastic simplifying assumptions are made about the underlying mechanism. In the following we use a theoretical model in which lamellar bodies, or a subfraction of them, are the sole immediate precursors of alveolar surfactant. The assumptions underlying this model have been detailed elsewhere (22,23). Briefly they amount to there being a steady-state compartmental precursor-product relationship between the two pools (lamellar bodies as the sole precursor and lavagable alveolar surfactant as product) being examined.

Under those circumstances, the mean residence time τ, for a molecular species in the product compartment is given by $\tau = V/\dot{Q}$, where V is the volume of the product compartment (*i.e.*, mg DPPC per g dry lung) and $\dot{Q}$ the flux of the molecular species through the product compartment (*i.e.*, mg DPPC per g dry lung per min, being the rate at which DPPC enters and leaves the alveolar compartment at steady state). As $\dot{Q}$ is usually unknown, τ is determined experimentally in one of two ways.

In the first, radiotracer is introduced intravenously as a precursor (*e.g.*, as [^{14}C]choline *via* a caudal vein) and the specific activity of the substance of interest (*i.e.*, DPPC) determined in precursor and product compartments. If $s_a(T)$ and $s_b(T)$ are the specific activities in precursor A and product B compartments, respectively, then provided a steady state precursor-product relationship exists, we have

$$s_b(T) = \int_{t=0}^{T} s_a(t)e^{(T-t)/\tau}\, dt \qquad [1]$$

and a direct least-squares estimate of τ may be obtained using, for instance, the Marquardt-Levenberg algorithm to minimize the residual error, and we have shown (13) that this least squares approach to the estimation of turnover time can be very much more efficient than several other methods in common use. Where several animals are used to determine the specific activity at each time point and when the data is homoscedastic, deviations from the regression model closely follow an F distribution, notwithstanding that the regression is nonlinear (14). This provides an objective basis on which to decide whether a precursor-product relationship might be presumed to exist between two compartments. Where the data is heteroscedastic, the equivalent test statistic, even in a linear model, does not follow an F distribution. The problem of hypothesis testing then becomes an extension of the well known Behrens-Fisher problem (24). In practice, the distribution of the test statistic is then most easily derived empirically using a Monte-Carlo simulation, which is the approach we have adopted. Unless the data is at least consistent with a precursor-product relationship, then there is not a theoretical basis on which to proceed to determine a turnover time in the putative product compartment.

Where the turnover of two different substances is being compared, there are four specific-activity time curves, two for each substance, and the aim is to compare the time-constants derived from each pair. Although mathematically straightforward, the approach precludes any meaningful "eyeballing" of the data, and the validity of the results is strongly dependent on knowing the correct precursor compartment for each of the two molecular species.

A second experimental approach to estimating τ is to introduce the tracer directly into the product compartment and to examine the fall of specific activity with time. In a compartmental process without recycling of tracer, the specific activity would fall exponentially toward zero with time constant $\tau = V/\dot{Q}$.

In practice however, the recycling of tracer complicates the picture so that the specific activity in the precursor compartment still enters the calculations. Initially, however, precursor specific activity is zero, and its effect is not dominant. It enters the equations only as a correction, the importance of which increases with time. For tracer introduced as a bolus into the product compartment at time zero, the product specific activity $s_b(T)$ is given by

$$s_b(T) = s_0 e^{-T/\tau} + \int_{t=0}^{T} s_a(t)e^{(T-t)/\tau}\, dt \qquad [2]$$

where s_0 is the product specific activity at time zero after the introduction of the bolus. As before, $s_a(t)$ is the precursor specific activity at time t.

The experimental data comprise, for each molecular species, precursor specific activities $A_{i,j}$, $i = 1, 2, \ldots, n$, $j = 1, 2, \ldots, m_{ai}$ there being n times at which specific activities were determined, and m_{ai} rats from which precursor specific activities were obtained at the i^{th} sample time. $B_{i,j}$ and m_{bi} are defined analogously for product specific activities. The calculations assume a zero precursor specific activity at time zero and a linear interpolation of precursor specific activity between sample times. Given that both precursor and product specific activities are subject to random error, we introduce as nuisance parameters to be estimated θ_i, $i = 1, 2, \ldots, n$ being precursor specific activity at time T_i, $i = 1, 2, \ldots, n$, and θ_0 the product specific activity at time zero. For notational convenience let $\bar{\theta} = (\theta_0, \theta_1, \ldots, \theta_n)$. Then $\bar{\theta}$ and τ are the parameters to be estimated. Given $(\tau, \bar{\theta})$, equation 2 gives the predicted product specific activities $P_i(\tau,\bar{\theta})$. Then we define as the objective function to be minimized

$$\Omega(\tau,\bar{\theta}) = \sum_{i,j} w_{a,i}[A_{i,j} - \theta_i]^2 + \sum_{i,j} w_{b,i}[B_{i,j} - P_i(\tau,\bar{\theta})]^2 \qquad [3]$$

where $w_{a,i}$ and $w_{b,i}$ are weights determined by the assumed error structure of the data. In heteroscedastic data

such as found here, the weights are the reciprocals of the variances found in the compartment and, at the time, appropriate to the data being weighted.

The calculation of the extent of recycling depends on the parameters estimated above. The radioactivity, R_0, initially in the product compartment is given by $R_0 = \theta_0 V$. The radioactivity entering from the precursor compartment, all of which is "recycled," is denoted and defined

$$R_r = \dot{Q} \int s_a(t)\, dt, \qquad [4]$$

from which, using $V/\dot{Q} = \tau$, the ratio of the two becomes

$$R_r/R_0 = \int s_a(t)/(\theta_0 \tau)\, dt \qquad [5]$$

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Hypolipidemic Effects of β-Cyclodextrin in the Hamster and in the Genetically Hypercholesterolemic Rico Rat

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The effect of increasing amounts of a cyclic oligosaccharide, β-cyclodextrin (BCD), included in the diet on plasma cholesterol and triglycerides, was investigated in two animal models, namely in male genetically hypercholesterolemic Rico rats and in male Syrian hamsters. The distribution of bile acids in the gastrointestinal tract and in the feces of hamsters was also determined. In the Rico rats and hamsters, plasma cholesterol and triglycerides decreased linearly with increasing doses of BCD. In these two species, 20% BCD as compared to control diet lowered cholesterolemia (−35%) and triglyceridemia (−70%). In the hamster, the BCD diet caused a marked decrease in cholesterol and triglycerides in chylomicrons and very low density lipoprotein, and in high density lipoproteins cholesterol. Composition and amounts of bile acids were modified in the gastrointestinal tract of hamsters receiving 10% BCD as compared to the control group. The total bile acid content of the gallbladder of treated hamsters was fourfold higher than in the control group, and the bile contained a large amount of hydrophilic bile acids. This trend was also observed in the small intestine, in which percentages and total quantities of cholic plus deoxycholic acids (cholic pathway) were higher than those of chenodeoxycholic plus ursodeoxycholic plus lithocholic acids (chenodeoxycholic pathway). The bile acid contents of the cecum and colon of treated hamsters were 2.7-fold higher than those of control animals, but the bile acid composition was similar in the two groups of hamsters. Fecal excretion of bile acids was 3.3-fold higher in the treated group than in the control group, and the percentage of lithocholic acid was markedly increased and close to that observed in the colon. The turnover of the chenodeoxycholic pool was twice as fast in treated hamsters as in control hamsters, whereas that of cholic acid was not significantly modified. These results suggest that BCD does not alter the microbial degradation of bile acids, but rather stimulates their synthesis and increases their pool size. BCD prevents the intestinal absorption of lithocholic acid and washes this cytotoxic bile acid from the colon. The hypocholesterolemic effect of BCD appears to be due to stimulation of bile acid synthesis.
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β-Cyclodextrin (BCD) is an enzymatically modified starch containing seven glucopyranose units all linked in a ring structure in α (1-4) position (1). BCD is cone-shaped with all of the secondary hydroxyl groups located at one end of the ring (the larger diameter end) and all of the primary hydroxyl groups at the other (2). The BCD cavity contains hydrogen and glycoside oxygen atoms and is therefore hydrophobic (3). BCD shows a remarkable ability to form inclusion complexes with various natural and synthetic molecules (4). In particular, BCD is able to encapsulate and solubilize cholesterol and bile acids to varying degrees *in vitro* (5-7). The inclusion process is influenced by the interactions between the guest molecules and the cavity of the host, and also by the shape and size of the guest. This can change the chemical and physical properties of the guest, such as stability, solubility and bioavailability.

When orally administered to animals for 13 weeks, BCD does not exert any toxic or genotoxic effects (8,9). BCD is not absorbed from the small intestine of rats (10). According to Gerloczi *et al.* (10), the cecum and colon of rats fed a BCD-containing diet contain large amounts of intact BCD, part of which is probably excreted in the feces. Whereas human salivary and pancreatic α-amylases do not hydrolyze this carbohydrate *in vitro* (11), it can be hydrolyzed by *Bacteroides* isolated from the human colon (12). BCD is slowly metabolized in the hindgut, probably by the microflora. The encapsulation of various molecules *in vivo* by BCD could modify the digestibility of the guest, but also of the host in the gastrointestinal tract.

In previous experiments we have observed that poorly digestible carbohydrates, such as lactose, amylomaize starch or pectin, modified bile acid metabolism and markedly reduced plasma cholesterol in germ-free or conventional rats (13-15). The mechanisms by which these carbohydrates exert their effects are poorly understood (16).

The purpose of the present study was to determine the effects of increasing levels of BCD, added to a semi-purified diet, on plasma cholesterol and triglyceride concentrations in genetically hypercholesterolemic Rico rats (17) and in Syrian golden hamsters. Subsequently, the effect of a diet containing 10% BCD on bile acid metabolism was analyzed in the male Syrian hamster which, like humans, possesses a gallbladder and shares a similar bile acid pattern (18).

MATERIALS AND METHODS

Animals and diets. The control semi-purified diet contained (g/kg): sucrose 625, lactic casein 200, lard 92, walnut oil 8, mineral mix 50, vitamin mix on cellulose 25, and cholesterol 0.5. In experimental diets, increasing amounts of BCD (Kleptose®, water content 12%, purity 99%, provided by Roquette Frères, Lestrem, France) were substituted for sucrose. The experimental diets contained 10, 50, 100 or 200 g of BCD/kg. Young male Syrian golden hamsters were purchased from the CNRS specialized breeding unit, Villejuif (France). Male hypercholesterolemic Rico rats were originally purchased from Ciba-Geigy (Basel, Switzerland).

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Abbreviations: BCD, β-cyclodextrin; C, control hamsters; CA, cholic acid; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HI, hydrophobicity index; K, chenodeoxycholic acid; LDL, low density lipoprotein; POPOP, 1,4-*bis*(5-phenyloxazol-2-yl)-benzene; PPO, 2,5-diphenyloxazole; T, 10% BCD treated hamsters; VLDL, very low density lipoprotein.

Experiment 1. Effect of BCD dose. Thirty hamsters and 18 Rico rats, 12-weeks-old at the beginning of the experiment, received food and water *ad libitum* throughout the 8 wk of the study. The room temperature was maintained at 23 ± 1°C and lighting conditions were controlled according to a 12-h cycle. Six hamsters per group were fed the control diet (C) or the experimental diet containing 1, 5, 10 or 20% BCD. Six rats per group were fed the control diet or this diet containing 10 or 20% BCD. During the last week of the experimental period, feces were collected for bile acid and neutral sterol analysis. At the end of the experiment, the hamsters and rats were killed at 10:00 a.m. by aortic puncture after Tiletamine-Zolazepam (Virbac, Paris, France) anesthesia. Plasma and liver were collected and immediately stored at −20°C. Two other groups of six hamsters each were used in order to determine lipoprotein composition. These hamsters, 12-weeks-old, received either the control diet or the experimental diet containing 20% BCD for 8 wk. After diethyl ether anesthesia, blood was collected over ethylenediaminetetraacetic acid (EDTA, 4%) and centrifuged at 3,000 × *g* for 15 min at 4°C. The plasma of two hamsters was pooled and 2 mL were used for lipoprotein isolation.

Experiment 2. Bile acid synthesis and distribution in the gastrointestinal tract and in the feces of hamsters. Two groups of four hamsters, 12-weeks-old, received either the control diet (C) or the experimental diet containing 10% BCD (T) for five weeks; thereafter they received these same diets containing 20,000 dpm/g of [24-^{14}C]chenodeoxycholic acid (CFA 533 batch 6, sp. act. 2,212 MBq/mmol, Amersham, Amersham, United Kingdom) for three weeks. Feces were collected from each hamster over two periods of four days during the last eight days of the experiment and were stored at −20°C. Finally, the hamsters were killed as previously described. Plasma, liver, gallbladder, small intestine, cecum and colon were collected and stored in ethanol prior to analysis.

Analysis of plasma cholesterol, triglycerides and lipoproteins. Total plasma cholesterol, triglycerides and phospholipids were determined enzymatically by using commercial reagent kits (Boehringer, Mannheim, Germany). For lipoprotein isolation, 2 mL of plasma was used and ultracentrifuged in a KBr density gradient at 105,000 × *g* for 24 h according to the method of Serougne *et al.* (19). Eighteen fractions were obtained, and cholesterol, triglycerides and phospholipids were determined in each fraction using commercial kits. Proteins were assayed by the method of Lowry *et al.* (20) using serum albumin as a standard.

Analysis of fecal sterols and bile acid distribution in the gastrointestinal tract. Lipids from the organs and feces were extracted with ethanol for 24 h in a Soxhlet apparatus. Lipid fractions were saponified in boiling ethanolic 2 M potassium hydroxide for 1 h. The sterols were extracted with hexane and bile acids were deconjugated by the method of Grundy *et al.* (21). Free bile acids were methylated with diazomethane, silylated with Deriva-sil (Chrompack, Middelburg, The Netherlands) and assayed on a Carlo Erba (Milano, Italy) HRGC 5160 gas chromatograph equipped with a standard fused silica WCOT capillary column cross-linked with OV1701 (Spiral, Dijon, France) or with CP Sil 5 CB (Chrompack) (length, 25 m; film thickness 0.2 μm; oven temperature, 240°C; flow-rate of hydrogen carrier gas, 2 mL/min).

For quantification of bile acids in the gastrointestinal tract and in the feces, an isotope equilibrium of [24-^{14}C]-chenodeoxycholic was used as described by Sacquet *et al.* (22). For three weeks, C and T hamsters received the radioactive diet. Dietary radioactive chenodeoxycholic acid absorbed daily in the intestine was diluted by newly synthesized or endogenous chenodeoxycholic acid until a constant dilution was obtained. When isotope equilibration was achieved, bile acids of the chenodeoxycholic pathway had the same specific activity. In particular, the specific activity of lithocholic acid (microbial metabolite of chenodeoxycholic), the most abundant fecal bile acid, became constant, and the input and output of the total radioactivity from the body were identical (see Results). The amounts of chenodeoxycholic acid and its metabolites in the gallbladder, small intestine, cecum, colon and feces were determined by dividing the radioactivities of the intestinal contents by the specific activity of lithocholic acid. These results and the percentages of chenodeoxycholic plus its microbial metabolites (chenodeoxycholic acid biosynthesis pathway, K) as determined by gas-liquid chromatography, allowed the calculation of the amounts of cholic acid plus its microbial metabolites (cholic acid biosynthesis pathway, CA) in the intestinal contents. The following equations were used:

$$\%\ \text{of CA} + \%\ \text{of K} = 100\% \quad [1]$$

$$\text{amount of K in } \mu\text{moles} = \frac{\text{radioactivity in the intestinal content}}{\text{specific activity of lithocholic acid in dpm} \times \mu\text{moles}^{-1}} \quad [2]$$

$$\text{amount of CA in } \mu\text{moles} = \frac{(\text{amount of K}}{\%\ \text{of K)}} \times (100 - \%\ \text{of K}) \quad [3]$$

Measurement of radioactivity. Radioactivities were measured in 2,5-diphenyloxazole (PPO)/1,4-*bis*(5-phenyloxazol-2-yl)benzene (POPOP) solution with a liquid scintillation spectrometer (MR 300 Kontron, Montigny le bretonneux, France).

Statistical analysis. Data are expressed as mean ± SEM and differences between groups were tested by ANOVA (Statview 512, Abacus Concepts Inc., Calabasas, CA).

RESULTS

When rats and hamsters received increasing doses of BCD up to 10% of the diet, body and liver weights, dietary intake and fecal excretion were no different from those of controls (Tables 1 and 2). With 20% BCD in the diet, liver weight was reduced by 22% and dietary intake increased by 15% in the hamster as compared to controls (Table 1). With this same diet, body weight was reduced by 12% in the rat as compared to controls (Table 2).

Concentrations of cholesterol and triglycerides. In the hamster, increasing doses of BCD in the diet decreased plasma cholesterol and triglyceride concentrations in a linear fashion. Regression lines were obtained; if y = plasma cholesterol concentration (mg/mL), or z = plasma triglyceride concentration (mg/mL), and x = % BCD in the diet, then $y = -0.028\ x + 1.583$ ($r = -0.811$, $P < 0.001$) and $z = -0.11\ x + 3.144$ ($r = -0.762$, $P < 0.001$).

TABLE 1

Data on Control Hamsters (C) and Hamsters Receiving Increasing Amounts of β-Cyclodextrin (BCD)

	Control[a]	BCD 1%	BCD 5%	BCD 10%	BCD 20%
Body weight[b] (12 wk) (g)	75.0 ± 3.5	78.2 ± 4.1	72.2 ± 3.6	74.5 ± 4.2	78.3 ± 3.8
Body weight (20 wk) (g)	88.6 ± 4.1	84.5 ± 2.2	83 ± 1.3	83.7 ± 1.2	88 ± 3.2
Liver weight (20 wk) (g)	4.6 ± 0.2	4.5 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	3.6 ± 0.1[c]
Food intake (g/d)	5.2 ± 0.10	5.2 ± 0.08	5.4 ± 0.10	5.2 ± 0.10	6.0 ± 0.05[c]
Fecal excretion (g/d)	0.26 ± 0.06	0.26 ± 0.04	0.24 ± 0.06	0.36 ± 0.01[d]	0.33 ± 0.01

[a]Mean ± SEM (n = 6).

[b]Values with different superscript letters are significantly different from the control group.

[c]$P < 0.05$.

[d]$P < 0.01$.

TABLE 2

Data on Control Rats and Rats Receiving Increasing Amounts of β-Cyclodextrin (BCD)

	Diet		
	Control[a]	BCD 10%	BCD 20%
Body weight[b] (12 wk) (g)	345 ± 15	350 ± 9	325 ± 15
Body weight (20 wk) (g)	461 ± 24.4	452 ± 9.4	406 ± 13.2[c]
Liver weight (20 wk) (g)	17.8 ± 0.9	19.4 ± 0.8	16.5 ± 0.7
Food intake (g/d)	21.3 ± 0.8	21.7 ± 0.5	20.0 ± 0.5

[a]Mean ± SEM (n = 5).

[b]Values with different superscript letters are significantly different from the control group.

[c]$P < 0.001$.

TABLE 3

Plasma Cholesterol and Triglyceride Concentrations (mg/mL) in Syrian Hamsters and Rico Rats Receiving Increasing Amounts of β-Cyclodextrin (BCD) in the Diet

Dietary groups	Syrian hamsters		Rico rats	
	Cholesterol[a]	Triglycerides	Cholesterol[a]	Triglycerides
Control[b]	1.53 ± 0.06	3.60 ± 0.30	1.67 ± 0.05	1.71 ± 0.32
BCD 1%	1.60 ± 0.04	2.70 ± 0.20[d]	N.D.[c]	N.D.[c]
BCD 5%	1.46 ± 0.09	2.70 ± 0.30[d]	N.D.[c]	N.D.[c]
BCD 10%	1.30 ± 0.10[e]	1.60 ± 0.10[f]	1.30 ± 0.03[e]	1.00 ± 0.15[d]
BCD 20%	1.02 ± 0.06[f]	1.20 ± 0.20[f]	1.00 ± 0.04[f]	0.80 ± 0.06[f]

[a]Mean ± SEM (n = 6 for hamsters, n = 5 for rats).

[b]Values with different superscript letters are significantly different from the control group.

[c]N.D., not determined.

[d]$P < 0.05$.

[e]$P < 0.01$.

[f]$P < 0.001$.

These decreases were also observed in Experiment 2. In the Rico rats, the decrease in plasma cholesterol and triglycerides was similar to that observed in the hamsters. Plasma cholesterol and triglycerides in the hamsters fed 20% BCD were, respectively 33 and 66% lower than in the control group, and 40 and 55% lower in the treated Rico rats than in the control Rico rats (Table 3). In the hamsters receiving 20% BCD in the diet as compared to the control group, plasma concentrations of cholesterol, triglycerides, phospholipids and proteins in chylomicrons plus very low density lipoproteins (VLDL) decreased by 70, 59, 69 and 67%, respectively (Table 4). The concentrations of these compounds were slightly decreased in LDL_2 (low density lipoproteins). In high density lipoproteins (HDL), the concentrations of cholesterol and phospholipids were also reduced by 26 and 20%, respectively, but the protein and triglyceride concentrations were unchanged.

Variations of fecal sterols in the hamsters receiving increasing levels of BCD. In the feces of control hamsters, the level of microbial metabolites of cholesterol, including major metabolites (coprostanol and cholestanol) and minor

TABLE 4

Plasma Lipoprotein Components (μg/mL) in the Hamsters Receiving the Control Diet or a Diet Containing 20% β-Cyclodextrin (BCD)

Plasma lipoprotein components	Groups	Chylo + VLDL[c] d < 1.006 μg/mL	LDL_1 1.006 < d < 1.040 μg/mL	LDL_2 1.040 < d < 1.063 μg/mL	HDL 1.063 < d < 1.21 μg/mL
Cholesterol[a]	Control[b]	259 ± 37	131 ± 14	148 ± 7	896 ± 25
	BCD 20%	76 ± 22[d]	112 ± 21	117 ± 20	663 ± 34[d]
Triglycerides	Control	2052 ± 204	334 ± 114	79 ± 13	143 ± 15
	BCD 20%	846 ± 150[d]	366 ± 5	54 ± 14	113 ± 3
Phospholipids	Control	612 ± 91	149 ± 4	158 ± 18	2224 ± 77
	BCD 20%	194 ± 35[e]	166 ± 42	135 ± 37	1790 ± 15[d]
Proteins	Control	336 ± 50	111 ± 22	167 ± 39	2932 ± 337
	BCD 20%	110 ± 6[d]	130 ± 6	117 ± 12	2970 ± 302

[a]Mean ± SEM (n = 3, pools of 2 hamsters).

[b]Values with different superscript letters are significantly different from the control group.

[c]Chylo + VLDL, chylomicrons plus very low density lipoproteins; LDL_1 and LDL_2, low density lipoproteins; HDL, high density lipoproteins.

[d]$P < 0.01$.

[e]$P < 0.001$.

TABLE 5

Percentages of Cholesterol and Its Microbial Metabolites in Feces and Daily Fecal Elimination of Neutral Sterols in Syrian Hamsters Receiving Increasing Amounts of β-Cyclodextrin (BCD)

	Cholesterol[a] (%)	Coprostanol (%)	Cholestanol (%)	Other sterols[b] (%)	Fecal elimination of neutral sterols (mg/day per hamster)
Control[c]	8.8 ± 1.3	72.0 ± 2.3	12.0 ± 0.8	7.1 ± 2.0	2.2 ± 0.4
BCD 1%	5.2 ± 0.7	75.6 ± 1.2	12.5 ± 1.1	6.6 ± 1.0	N.D.[d]
BCD 5%	31.1 ± 2.2[e]	52.1 ± 2.0[e]	12.6 ± 2.5	4.1 ± 0.9	N.D.
BCD 10%	29.9 ± 6.5[f]	50.6 ± 6.2[f]	15.0 ± 0.7[e]	4.3 ± 1.1	2.7 ± 0.7
BCD 20%	39.7 ± 4.2[g]	48.3 ± 3.4[g]	9.6 ± 0.5[e]	2.3 ± 0.7[e]	7.0 ± 0.9[g]

[a]Mean ± SEM (n = 6).

[b]Other sterols, epicoprostanol, cholestanone, cholestenone and others.

[c]Values with different superscript letters are significantly different from the control group.

[d]N.D., not determined.

[e]$P < 0.05$.

[f]$P < 0.01$.

[g]$P < 0.001$.

TABLE 6

Composition of Fecal Bile Acids in the Hamsters Receiving the Control Diet or a Diet Containing Increasing Amounts of β-Cyclodextrin (BCD)

	Bile acids					
Dietary groups	Cholic[a] (%)	Deoxycholic (%)	Chenodeoxycholic (%)	Ursodeoxycholic[b] (%)	Lithocholic (%)	Ketones (%)
Control[c]	1.9 ± 0.4	40.0 ± 1.5	4.5 ± 0.8	7.6 ± 5.8	34.8 ± 3.4	10.6 ± 1.4
BCD 1%	5.6 ± 4.9	38.9 ± 3.1	2.0 ± 0.5	4.8 ± 2.2	40.7 ± 3.5	5.4 ± 1.1
BCD 5%	3.0 ± 2.9	38.1 ± 8.8	1.6 ± 0.1	6.0 ± 1.7	45.8 ± 10.3	5.5 ± 2.2
BCD 10%	1.1 ± 0.3	36.4 ± 3.6	0.4 ± 0.2	3.9 ± 0.8	53.8 ± 2.6[d]	5.1 ± 0.6
BCD 20%	4.3 ± 0.6	35.8 ± 0.8	0.1 ± 0.1[d]	2.5 ± 1.4	54.4 ± 3.2[d]	2.4 ± 1.3[d]

[a]Mean ± SEM (n = 6).

[b]Identified by gas-liquid chromatography on OV 1701 and on CPSIL 5 CB capillary columns.

[c]Values with different superscript letters are significantly different from the control group.

[d]$P < 0.05$.

metabolites (epicoprostanol, cholestanone and cholestenone, called "other sterols" in Table 5), was ninefold higher than that of cholesterol itself. The addition of 1% BCD to the diet did not modify the sterol pattern, while 5, 10 or 20% BCD in the diet induced a much higher proportion of cholesterol and lowered the percentages of microbial sterols. The daily fecal elimination of neutral sterols was threefold higher in hamsters fed 20% BCD than in controls. Thus, BCD reduced microbial transformation of cholesterol and increased its fecal output and that of its by-products (Table 5).

The composition of bile acids in the feces was only slightly modified by increasing levels of BCD in the diet (Table 6). However, the percentages of lithocholic acid increased, while those of chenodeoxycholic acid decreased when the level of BCD in the diet was elevated. The increased proportion of lithocholic acid in the feces of hamsters receiving 10 or 20% BCD led us to compare the amount and nature of bile acids along the gastrointestinal tract and in the feces, in hamsters fed 10% BCD in the diet and in controls.

Distribution of bile acids in the gastrointestinal tract and in the feces of control hamsters (C) and treated hamsters (T) receiving 10% of BCD. In the gallbladder, bile acid content was 4.7-fold higher in the T group than in the C group (Table 7). In both groups, primary bile acids accounted for almost 75% of the total bile acids. The percentage of CA was increased, whereas the percentages of chenodeoxycholic acid and lithocholic acid were decreased in the T group as compared to the C group. In the small intestine, the bile acid content tended to be higher in the T than in the C group, but the bile acid composition was similar to that observed in the gallbladder, except for lithocholic acid and ketones which were increased in the two groups. In the cecum, bile acid content also tended to be higher in the T than in the C group. In both groups, the primary bile acids disappeared and microbial bile acids or secondary bile acids accounted for 95% of total bile acids. The compositions of these acids were similar and 7-oxolithocholic plus lithocholic acids accounted for 55–70% of total bile acids in the two hamster groups. In the colon, bile acid content was 2.7-fold higher in the T than in the C group, the bile acid composition being similar in these two groups. However, in comparison to what was observed in the cecum, 7-oxolithocholic acid had virtually disappeared, and the percentage of lithocholic acid in the colon corresponded to the sum of the percentages of 7-oxolithocholic and lithocholic acids in the cecum. Daily fecal elimination of bile acids was 3.3-fold higher in the T than in the C group. The fecal composition in both groups was virtually the same; 7-oxolithocholic acid was only present in trace amounts, but the percentage and mass of lithocholic acid were 1.6- and 5.3-fold higher, respectively, in the T than in the C group.

Pool sizes, biosynthesis and turnover of bile acids in the control and BCD treated hamsters. Bile acid pool sizes were calculated as the sum of bile acids in each part of the gastrointestinal tract, and the daily turnover of these acids was calculated as the ratio of bile acid pool size to daily fecal elimination of bile acids. When the isotope equilibrium of [24-^{14}C]chenodeoxycholic acid was achieved, the specific activity of lithocholic acid remained constant. It was determined in the extract of feces collected during two periods and corresponding to the 15–18th (a)

TABLE 7

Composition of Bile Acids in Various Parts of the Gastrointestinal Tract in Hamsters Receiving the Control Diet or a Diet Containing 10% β-Cyclodextrin (BCD)

Organs	Groups	Bile acids						Bile acids content (μmoles)
		Cholic[a] (%)	Deoxycholic (%)	Chenodeoxycholic (%)	7-Oxolithocholic (%)	Lithocholic (%)	Ursodeoxycholic[b] (%)	Ketones[c] (%)	
Gallbladder	Control[d]	49.9 ± 2.7	7.1 ± 0.9	25.9 ± 3.5	0.0	11.0 ± 2.6	1.6 ± 0.6	3.9 ± 0.8	1.0 ± 0.2
	BCD 10%	67.4 ± 0.8[e]	10.6 ± 1.1	11.6 ± 2.2[e]	0.0	3.3 ± 0.4[e]	3.3 ± 1.7	3.7 ± 0.5	4.7 ± 0.4[e]
Small intestine	Control	36.2 ± 4.8	1.6 ± 0.6	5.4 ± 1.1	1.2 ± 0.3	38.2 ± 2.8	1.8 ± 0.1	15.3 ± 7.0	5.4 ± 0.6
	BCD 10%	53.8 ± 4.7	1.0 ± 0.2	2.3 ± 0.6	1.0 ± 0.2	26.7 ± 4.1	0.6 ± 0.2[e]	14 ± 4.8	10.3 ± 2.4
Cecum	Control	1.0 ± 0.2	13.4 ± 5.6	2.2 ± 0.5	30.8 ± 2.9	38.2 ± 7.8	3.7 ± 1.1	11.5 ± 2.1	1.4 ± 0.4
	BCD 10%	2.7 ± 0.9	24.8 ± 4.1	2.7 ± 1.4	22.3 ± 2.7	32.9 ± 1.1	2.9 ± 0.8	10.4 ± 1.4	3.8 ± 1.2
Colon	Control	2.0 ± 0.6	11.7 ± 1.1	0.6 ± 0.1	4.0 ± 0.7	68.4 ± 3.5	11.1 ± 1.1	2.0 ± 0.6	1.8 ± 0.3
	BCD 10%	1.6 ± 0.5	14.6 ± 2.0	0.1 ± 0.1[f]	4.5 ± 0.6	73.6 ± 2.4	2.8 ± 0.7[e]	1.3 ± 1.0	4.9 ± 0.8[f]
Feces	Control	6.3 ± 2.1	30.9 ± 2.1	2.9 ± 0.8	1.8 ± 0.8	36.2 ± 3.5	13.2 ± 3.0	8.5 ± 1.1	μmoles/day 2.5 ± 0.09
	BCD 10%	0.1 ± 0.1[g]	32.1 ± 3.9	2.7 ± 1.1	0.1 ± 0.1	58.4 ± 3.9[f]	6.7 ± 0.8	0.1 ± 0.1[g]	8.2 ± 0.7[g]

[a] Mean ± SEM (n = 4).
[b] Identified by GLC on OV1701 and on CP SIL 5 CB capillary columns.
[c] Ketones: 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid, 3α,12α-dihydroxy-12-oxo-5β-cholanoic acid and unknown peaks.
[d] Values with different superscript letters are significantly different from the control group.
[e] $P < 0.01$.
[f] $P < 0.05$.
[g] $P < 0.001$.

TABLE 8

Pool Size (μmoles), Biosynthesis Rate and Turnover Time of Chenodeoxycholic and Cholic Acids in Hamsters Receiving the Control Diet or a Diet Containing 10% β-Cyclodextrin (BCD)

	Biosynthetic pathway	Control[a]	BCD 10%
Pool size[b] (μmoles)	Chenodeoxycholic	5.90 ± 0.40	11.00 ± 0.70[c]
	Cholic	3.80 ± 0.20	12.70 ± 1.00[d]
Biosynthesis rate (μmoles/day)	Chenodeoxycholic	1.50 ± 0.07	5.70 ± 0.30[d]
	Cholic	1.04 ± 0.06	2.50 ± 0.30
Turnover time (d)	Chenodeoxycholic	3.90 ± 0.30	1.90 ± 0.02[d]
	Cholic	3.70 ± 0.40	4.90 ± 0.80

[a]Mean ± SEM (n = 4).

[b]Values with different superscript letters are significantly different from the control group.

[c]$P < 0.001$.

[d]$P < 0.01$.

and 19–22nd (b) days of the experiments. In the T group, this specific activity was (a) 17,290 ± 1,620 dpm/μmol and (b) 16,630 ± 900 dpm/μmol. In the C group, this specific activity was (a) 45,460 ± 3,560 dpm/μmol and (b) 38,709 ± 1,200 dpm/μmol. Moreover, the input and output of radioactivity in the two groups were almost identical. Under these conditions, the biosynthesis of bile acids was determined by the daily fecal elimination of the acids. The chenodeoxycholic acid and CA pool sizes were, respectively, 1.8- and 3.3-fold higher in the T than in the C group (Table 8). The pool size of chenodeoxycholic acid was greater than that of CA in the C group, whereas this difference disappeared in the T group. The biosynthesis of bile acids *via* the chenodeoxycholic pathway or the cholic pathway were, respectively, 3.8- and 2.6-fold higher in the T than in the C group. In the first group, the level of bile acids of the chenodeoxycholic acid synthesis pathway was twofold higher than that of the CA synthesis pathway; in contrast, these two pathways were almost identical in the C group. Thus, the turnover of bile acids of the chenodeoxycholic acid pathway in the T group was half that observed in the C group, whereas the turnover of bile acids of the CA pathway was not significantly altered.

DISCUSSION

BCD did not modify the physiological status (*i.e.*, growth, dietary intake, body and liver weights, etc.) of the hamsters and rats when it represented less than 10% of the diet. Similar results have been obtained with rats which ingested 1.25–10% BCD in the diet for 13 wk (9). When the BCD level reached 20%, the dietary intake of the hamster increased, but body weight was not changed; with rats body weight decreased slightly. A similar decrease in body weight in dogs and rats fed high levels of BCD in the diet has also been observed (23). The weight loss could be explained by the poor digestibility of this carbohydrate, which is not absorbed in the small intestine and only partially fermented by the intestinal microflora (10).

BCD induced a marked decline in plasma triglyceride and cholesterol levels in the hamster and in the genetically hypercholesterolemic rat, which reflected the decline in the triglyceride-rich particles and HDL. This hypolipidemic effect was linearly related to increased dietary BCD intakes. Several patents have claimed that the hypocholesterolemic effect of BCD and its chemical derivatives is "well-known" (24,25). However, the hypocholesterolemic effect of dietary BCD was first reported in 1990 by Pitha (26). The existence of an inclusion complex between cholesterol and BCD was indicated in 1982 (27); yet, tangible proof for the inclusion of cholesterol in BCD has only been obtained recently (6). Taken together, this suggests that the hypolipidemic effects of BCD are still poorly understood. In the present experiments, the BCD effects observed may have resulted from at least two changes that occurred in the metabolism of cholesterol and triglycerides, namely from a decrease in intestinal absorption of dietary cholesterol and triglycerides, and a stimulation of the catabolism of cholesterol into bile acids.

The decrease in the intestinal absorption of cholesterol by dietary BCD in hamsters is supported by two observations. Firstly, fecal elimination of cholesterol and its byproducts was 3.2-fold higher in the hamsters receiving 20% BCD in the diet than in the control group. Secondly, the dietary intake was only 15% higher in the first group than in the control; this small increase could not explain the stimulation of the fecal elimination of cholesterol. The decrease in cholesterol absorption was probably linked to the encapsulating capacity of BCD for cholesterol (6) and/or to disruption of intestinal bile salt mixed micelles following bile acid complexation. The complexation with BCD has been observed *in vitro* for various bile acids in aqueous medium (5,7). Moreover, the microbial transformation of cholesterol, which normally occurs in the cecum and colon, was markedly reduced by the diets containing 10 and 20% BCD. This also suggests that an intense cholesterol/BCD interaction occurs in these intestinal compartments. Such an interaction could be induced either by cholesterol encapsulation, which would prevent its microbial degradation, or by a change in the composition of the BCD fermenting microflora, which may eliminate the bacteria responsible. In the rats receiving poorly digestible carbohydrates (lactose, raw potato starch), a linear decrease of the transformation of cholesterol into coprostanol has also been observed (28).

In the hamsters, dietary BCD strongly stimulated bile acid synthesis, as determined by fecal excretion of the acids (+360%). The encapsulation of bile acids by BCD in the intestine probably prevents their reabsorption and

consequently interrupts their enterohepatic circulation, promoting their fecal elimination. When the enterohepatic circulation of bile acids is interrupted either by biliary bile diversion or by binding to a resin within the intestinal lumen, transport of bile acids to the hepatocyte is lowered and their synthesis *via* cholesterol 7α-hydroxylase is stimulated (29). These processes are altered due to the affinity of bile acids for BCD. The stimulation of biosynthesis is greater for the chenodeoxycholic acid pathway, expressed by formation of microbial metabolites (mainly lithocholic acid) in feces, than for the CA pathway. The encapsulation process is related to the hydrophobicity index (HI) of these bile acids. The HI of sodium glycochenodeoxycholate and glycolithocholate (+0.51 and 1.05, respectively) was higher than that of sodium glycocholate (+0.07) (30). *In vitro*, the affinity of free bile acid or glycine conjugate of chenodeoxycholic acid for BCD is higher than that of CA (5,7). The encapsulation of chenodeoxycholic acid in the small intestinal lumen probably increased its input into the cecum, where the bile acid can be degraded by the microflora to 7-oxolithocholic and lithocholic acids. The diet containing BCD, as compared to the control diet, did not modify the microbial pattern of bile acid degradation in the cecum and colon, but increased their intestinal contents. This microbial pattern was also observed in the hamster receiving chenodeoxycholic acid in its diet (31). The marked decrease of 7-oxolithocholic acid in the colon and its absence in the feces of the C and T hamsters suggests either its microbial transformation into lithocholic acid or its colonic reabsorption, the latter being supported by the observation reported in the literature of the absorption of 7-oxolithocholic acid through the rat colon (32). Furthermore, BCD prevented the absorption of lithocholic acid in the colon, the percentage and the amount of lithocholic acid in the feces as compared to those in the colon being significantly higher in the T than in the C group. This leads to the assumption that the very strongly hydrophobic lithocholic acid is still encapsulated in the unfermented colonic BCD (10). Under these conditions, BCD could prevent the cytotoxic effect and colonic mucosal damage induced by lithocholic acid (33).

BCD also increased the intestinal bile acid pool sizes. Previous work by Riottot and colleagues has shown that poorly digestible carbohydrates increase bile acid pool size in rats (14), and that this increase was correlated to a stimulation of bile acid absorption in the ileum (16). This process also could be modulated according to the bile acid affinity for BCD. Consequently, the turnover of chenodeoxycholic acid in the gastrointestinal tract was markedly decreased in the T group as compared to the C group of hamsters and the life-span of lithocholic acid in the colon was significantly reduced.

In the genetically hypercholesterolemic rats, the hypolipidemic action of BCD was also very pronounced and could have resulted from similar modifications as those observed in the BCD-treated hamsters. In genetically hyperlipidemic humans, the reduction in plasma cholesterol and triglycerides by dietary manipulation is generally moderate (34).

The effects of BCD were much more interesting than those induced by the classical bile acid binding resin cholestyramine. In contrast to this resin, BCD decreased plasma triglycerides, specifically stimulated the chenodeoxycholic acid pathway of biosynthesis, increased the bile acid pool (antilithiasic effect), eliminated a cytotoxic bile acid rapidly and specifically, eliminated dietary and endogenous cholesterol in feces, and was probably digested (energy production) by the microflora (35,36). Other poorly digestible carbohydrates, such as amylomaize starch, pectin or guar gum, also decreased plasma cholesterol (14,37,38), although their lipid lowering actions are poorly understood.

In conclusion, the hypolipidemic effect of BCD was shown to be due to the stimulation of bile acid biosynthesis and to the decrease of cholesterol absorption, the former being one of the most important mechanisms in the elimination of cholesterol from the plasma and, more generally from the body in the rat, hamster and humans (18,39,40). Given the efficacy of BCD observed in the two different species which we examined in the present series of experiments, it may be possible, and of interest, to carry out further studies on the hypolipidemic effects of BCD in humans.

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The Effect of Culture Medium Composition on Ether Lipid Cytotoxic Activity

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The effect of a serum-free medium (TNB-100), compared to RPMI 1640 containing 10% fetal bovine serum (FBS), on the lipid composition of HL60 and K562 leukemic cells was investigated. The 10% FBS RPMI medium contained approximately three times more phospholipids (PL), about three times more protein and eight times more cholesterol (CHOL) than did the TNB-100 medium. Cells cultured in TNB-100 medium, referred to as HL60-TNB and K562-TNB cells, were significantly lower in PL and CHOL than 10% FBS RPMI cells, with about a threefold higher PL-to-CHOL ratio; however, these cells were significantly higher in protein content. Cells grown in TNB-100 were also significantly more fluid than 10% FBS RPMI cells and were more sensitive to the fluidizing action of the ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine. The 50% inhibitory dose of the drug was about 50% lower in TNB-grown cells than in 10% FBS RPMI cells.

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Synthetic alkylglycerophospholipids, also known as ether lipids (EL), are a new class of agents that are used for the experimental therapy of cancer and leukemia (1-3). They are known to interact with the plasma membrane of neoplastic cells and to modify membrane biochemical (4-8) and biophysical properties (9-11). EL have been shown to exert a direct and specific effect on neoplastic cells; they cause preferential growth inhibition of both mouse and human leukemic cells as compared to normal bone marrow progenitor cells. These observations have led to clinical trials of EL as selective bone marrow purging agents (12,13). In addition, EL are cytotoxic, have antiinvasive capacity and induce malignant cell differentiation (1,3).

The molecular mechanisms leading to these direct effects are still not clear. Malewicz and Baumann (14) have shown first that membrane cholesterol (CHOL) levels affect the selective toxic action of EL. Recent data from our group (15,16) indicate that CHOL content modulates EL membrane effects and toxicity in leukemic cells. In fact, cells resistant to the toxic action of EL, such as K562 leukemic cells (17), are also richer in CHOL than sensitive HL60 cells (16). Moreover, by depriving K562 cells of their membrane CHOL content, it is possible to make them sensitive to originally nontoxic doses of EL (18).

The CHOL concentration in the culture medium is related to the cytotoxic action of EL, suggesting that the CHOL from serum added to the medium may modulate the biological activities of these drugs (16). This may be due, in part, to different growth conditions which may modify membrane lipid composition, or to a direct interaction of CHOL in the medium with EL resulting in reduced drug availability to the cells. We have suggested (18) that membrane CHOL levels should be considered when exposing cancer cells to EL *in vitro*, as for bone marrow purging, with the aim of optimizing the sensitivity of residual leukemic cells to EL. For this purpose we also investigated whether the use of a defined serum-free medium would affect CHOL levels in leukemic cells and consequently EL membrane effects and toxicity.

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Abbreviations: CHOL, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; EL, ether lipids; FBS, fetal bovine serum; ET-18-OMe, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ID_{50}, 50% inhibitory dose; PBS, phosphate buffered saline; PL, phospholipid(s).

MATERIALS AND METHODS

Chemicals. The ether lipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe) was provided by Dr. R. Nordstrom, Medmark Pharma (Munich, Germany). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Janssen (Beerse, Belgium). Erythrosin B (tetraiodofluorescein) was purchased from BDH (Poole, England). TNB basal medium with L-glutamine (2 mM) and lipid/protein complex were obtained from Seromed-Biochrom KG (Berlin, Germany). TNB-100 medium, obtained from 20 mL of lipid/protein complex and 980 mL of TNB basal medium, is defined as serum-free medium (19). The composition of the lipid/protein complex has been described (19). All other reagents were of analytical grade.

Cells. Human leukemia HL60 and K562 cells were obtained from Istituto Zooprofilattico Sperimentale (Brescia, Italy) and were free of mycoplasma contamination. Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 2mM L-glutamine, or in TNB-100 serum-free medium at 37°C in a humidified atmosphere containing 5% CO_2. Cells cultured in TNB-100 medium were defined as HL60-TNB and K562-TNB.

Phospholipid and cholesterol determinations. Lipids were extracted from 5 mL of 10% FBS RPMI or TNB-100 medium, or from the cell suspension (10×10^6 cells, total) according to Folch *et al.* (20). Phosphorus of total phospholipids (after acidic digestion) and cholesterol were determined according to Svanborg and Svennerholm (21).

Membrane fluidity. Membrane fluidity was assessed in cell suspension according to Shinitzky and Barenholz (22) using DPH as fluorescent probe. In order to measure the fluidizing effect of ET-18-OMe, cells cultured in 10% FBS RPMI or in TNB-100 medium were incubated with 10 μM ET-18-OMe for 2 h; cell viability was 100% at the end of the incubation. Control cells were incubated in the same volume of vehicle. Cells were then washed twice with Dulbecco's phosphate-buffered saline (PBS) and resuspended at $1\text{-}2 \times 10^5$/mL in PBS containing DPH (10^{-6} M). The mixture was incubated at 37°C for 30 min, and the fluorescence polarization (*P* values) were measured at 25°C using an MV-1 microviscosimeter (Elscint, Haifa, Israel). *P* values are a direct assessment of membrane fluidity as previously described (16). Arrhenius plots were

obtained for the cells with microviscosity (μ) being measured from 17 to 41°C. For each curve, the activation energy (ΔE) was determined according to Shinitzky and Barenholz (22) according to the equation: $\log \eta = \log A + \Delta E/RT$, where A is the value of the intercept of the curve with the ordinate, R is a rotational diffusion constant which was taken to be 0.362 (23) and T is the temperature expressed in degrees Kelvin.

Protein determination. Protein concentrations in 10% FBS RPMI and TNB-100 media or in the cells (after freezing and thawing) were measured according to Lowry *et al.* (24).

Cytotoxicity. EL cytotoxicity against HL60, HL60-TNB, K562 and K562-TNB cells was measured by erythrosin B dye exclusion. Different concentrations (0.5–40 μM) of ET-18-OMe were incubated with 5×10^5 cells/mL in 24-well culture dishes. After 2, 24 and 48 h at 37°C in the presence of 5% CO_2 and 95% humidified air, viable cells, *i.e.* those that retained a permeability barrier against erythrosin B, were counted.

Statistical analysis. Duncan's test for multiple comparisons was used.

RESULTS

Table 1 lists the phospholipid (PL), CHOL and protein content of the two media used for growing leukemic cells: RPMI 1640 containing 10% FBS and the serum-free TNB-100. The 10% FBS RPMI medium contained about three times more PL, three times more protein and 8 times more CHOL than TNB-100. We define cells cultured in normal 10% FBS RPMI medium as HL60 and K562 and cells grown in TNB-100 medium as HL60-TNB and K562-TNB.

TABLE 1

Phospholipids, Cholesterol and Protein Content of 10% Fetal Bovine Serum (FBS) RPMI 1640 and TNB-100 Media[a]

	Cholesterol (μg/mL)	Phospholipid (μg/mL)	Protein (mg/mL)
10% FBS RPMI	8.31 ± 0.1	0.99 ± 0.1	7.77 ± 0.3
TNB-100	1.10 ± 0.1[b]	0.31 ± 0.1[b]	2.62 ± 0.2[b]

[a] Each value is the mean ± SD of three different experimental values, obtained on three different samples.

[b] $P \leq 0.01$ according to Duncan's test for multiple comparisons.

Table 2 shows the protein content and the lipid composition of cells grown in the two media. The protein content was higher in leukemic cells cultured in TNB-100 medium (HL60-TNB and K562-TNB) than in HL60 and K562 cells cultured in 10% FBS RPMI. The TNB cells had significantly lower PL and CHOL contents, PL being 37% and CHOL being 81% lower in HL60-TNB than in HL60 cells. The reductions in K562-TNB cells were 63 and 88% respectively, compared with K562 cells. In both cell lines cultured in TNB-100 medium, there was a significant increase (about 3-fold) of the PL-to-CHOL ratio (PL/CHOL). As we have previously shown K562 cells are richer in CHOL than HL60 cells (16,18).

Figure 1 shows the relationship between the reciprocal temperature and the logarithm of microviscosity (η) for HL60 and HL60-TNB cells (Panel A) and for K562 and K562-TNB cells (Panel B). The cell microviscosity was measured in the range from 17 to 41°C. The equations describing each curve were: (i) for HL60 cells, $\log \eta = 0.917 - \log 2.834$ and the correlation coefficient was $r = 0.99$; (ii) for HL60-TNB cells, $\log \eta = 0.795 - \log 2.527$ and $r = 0.99$; (iii) for K562 cells, $\log \eta = 1.244 - \log 3.863$ and $r = 1.00$; (iv) for K562-TNB cells, $\log \eta = 1.269 - \log 4.044$ and $r = 0.99$. The temperature profiles of the four curves did not show any gel-to-liquid crystalline phase transition.

For each of these curves the activation energy (ΔE) was established at different temperatures (22). The ΔE values calculated at 25 and 37°C were: for HL60 cells, 2.73 and 2.85 Kcal/mol; for HL60-TNB cells, 2.37 and 2.46 Kcal/mol; for K562 cells, 3.71 and 3.82 Kcal/mol; for K562-TNB cells, 3.78 and 3.94 Kcal/mol.

Changes in EL toxicity related to lipid composition were investigated by incubating the cells with different doses of ET-18-OMe for 2, 24 and 48 h (Table 3). The 50% inhibitory doses (ID_{50}) were about 50% lower in HL60-TNB cells than in HL60 cells ($P \leq 0.01$). A similar enhancement of inhibition was seen for K562-TNB cells compared with K562 cells. Table 4 lists the fluorescence polarization data. Cells cultured in TNB-100 medium were significantly more fluid than cells cultured in 10% FBS RPMI. HL60 cells are known to be sensitive to the fluidizing action of EL, with an increase in fluidity of about 7% occurring after exposure to 5 μM EL for 2 h (16). In HL60-TNB cells the same dose of ET-18-OMe lowered the bulk fluidity by 14%. K562 cells, originally insensitive to the fluidizing action of EL (18), became sensitive when cultured in TNB-100 medium, showing a reduction in

TABLE 2

Lipid Composition of HL60 and K562 Leukemic Cells Cultured in 10% Fetal Bovine Serum RPMI 1640 or in Serum-Free TNB-100 Medium[a]

Cell type	Phospholipids (μmol/mg protein)	Cholesterol (μmol/mg protein)	PL/CHOL ratio	Protein (mg/10^6 cells)
HL60	1.140 ± 0.01	0.366 ± 0.05	3.11	0.034 ± 2
HL60-TNB	0.712 ± 0.01[c]	0.067 ± 0.01[c]	10.62[c]	0.047 ± 3[b]
K562	0.784 ± 0.02	0.416 ± 0.02	1.88	0.062 ± 3
K562-TNB	0.284 ± 0.01[c]	0.046 ± 0.01[c]	6.17[c]	0.172 ± 6[c]

[a] Each value is the mean ± SD of at least five determinations. PL, phospholipids; CHOL, cholesterol. HL60-TNB and K562-TNB = cells cultured in serum free TNB-100 medium.

[b] $P \leq 0.05$ and [c] $P \leq 0.01$ according to Duncan's test for multiple comparisons.

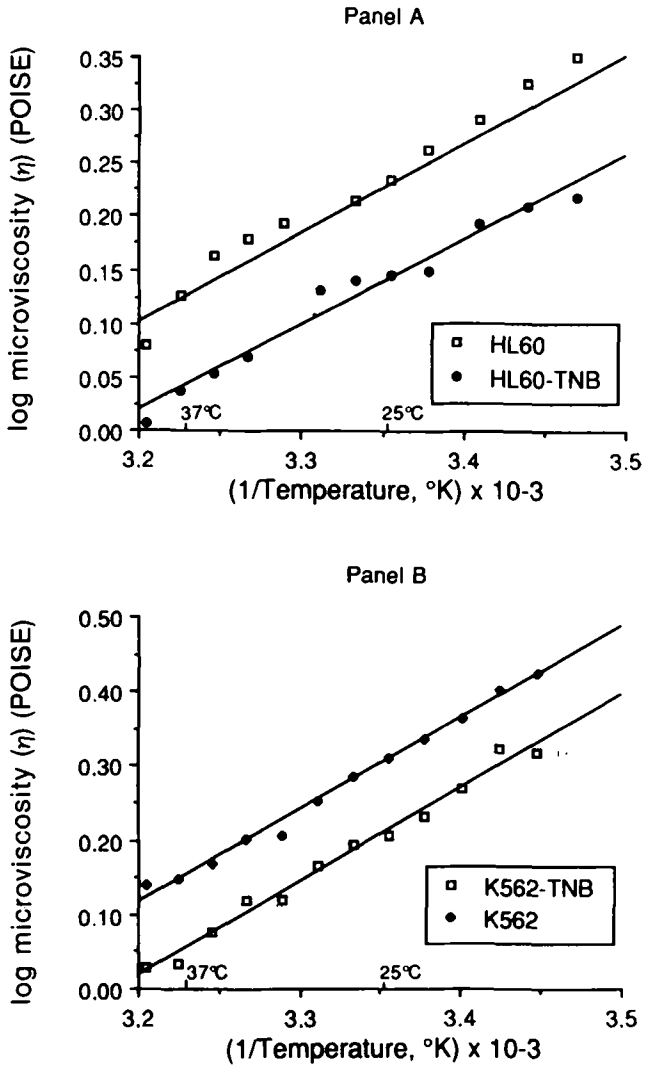

FIG. 1. Arrhenius plots for HL60 and HL60-TNB cells (Panel A) and for K562 and K562-TNB cells (Panel B). Each point is the mean of three experimental values.

fluorescent polarization of about 6% after exposure to 5 μM ET-18-OMe. Cell viability was 100% at the end of incubation.

DISCUSSION

Our recent data indicated that a correlation exists between the PL/CHOL ratio of the cell membrane and the cytotoxic effect of EL on leukemic cells (16). Modulation of the PL/CHOL ratio of leukemic cells, by extracting CHOL from the cell membrane, has been proposed as a method for enhancing the effectiveness of EL (18).

The PL/CHOL ratio of the cell membrane may also be changed by changing growth conditions, for example, by using media containing different percentages of serum and consequently of CHOL (16). We have here investigated whether the PL/CHOL ratio of HL60 and K562 leukemic cells which are, respectively, sensitive and quite resistant to EL toxic action (16,18), can be modified by culturing the cells in TNB-100 medium, a defined serum-free medium which contains about three times less PL and

TABLE 3

Effect of Culture Medium on ET-18-OMe 50% Inhibitory Doses (ID_{50})[a]

Cell type	ID_{50} (μM) 2 h	24 h	48 h
HL60	60.0 ± 4	4.1 ± 0.5	2.5 ± 0.5
HL60-TNB[b]	23.0 ± 5	2.4 ± 0.5	1.2 ± 0.5
K562	108.8 ± 5	36.4 ± 2	11.9 ± 0.7
K562-TNB[b]	69.9 ± 3	18.5 ± 3	4.4 ± 0.3

[a]Each value is the mean ± SD of at least four different experimental values obtained on three different samples. HL60 and K562, cells cultured in RPMI 1640 10% fetal bovine serum (FBS) medium. HL60-TNB and K562-TNB, cells cultured in serum-free TNB-100 medium.

[b]$P \leq 0.01$ *vs.* HL60 or K562 cells cultured in 10% FBS RPMI.

TABLE 4

Fluidizing Action of ET-18-OMe on HL60 and K562 Leukemic Cells Cultured in 10% FBS RPMI 1640 and Serum-Free TNB-100 Medium[a]

Cell type and additions	*P* value (25°C) ± SD
HL60	0.2100 ± 0.001
HL60 + ET-18-OMe	0.1950 ± 0.001[c]
HL60-TNB	0.1725 ± 0.001[c]
HL60-TNB + ET-18-OMe	0.1482 ± 0.003[c,d]
K562	0.2045 ± 0.001
K562 + ET-18-OMe	0.2050 ± 0.001
K562-TNB	0.1960 ± 0.004[b]
K562-TNB + ET-18-OMe	0.1847 ± 0.001[c,d]

[a]Each value is the mean ± SD of at least six determinations. HL60 and K562, cells cultured in RPMI 10% fetal bovine serum (FBS) medium. HL60-TNB and K562-TNB, cells cultured in serum-free TNB-100 medium. ET-18-OMe, 5 μM ET-18-OMe for 2 h. Viability was 100% at the end of incubation.

[b]$P \leq 0.05$ and [c]$P \leq 0.01$ *vs.* HL60 or K562 cells cultured in 10% FBS RPMI; and [d]$P \leq 0.01$ *vs.* HL60-TNB or K562-TNB cells, according to Duncan's test for multiple comparisons.

protein and 8 times less CHOL than 10% FBS RPMI. The growth of leukemic cells in this medium, leading to HL60-TNB and K562-TNB cells, significantly modified the plasma membrane PL/CHOL ratio, increasing it by about three times compared to cells cultured in 10% FBS RPMI (Table 2). The cells grown in TNB-100 medium are also richer in protein relative to the cells cultured in 10% FBS RPMI (Table 2). Because the serum-free medium and the serum-containing medium have different compositions, the differences in sensitivity to the cytotoxic action of ET-18-OMe could not be attributed to the differences between PL/CHOL ratios alone. TNB-100 medium also contains 2 g/L of albumin which may bind lipids and thus modify the uptake of ether lipids (25). We measured the uptake of [^{3}H]ET-18-OMe in HL60 and K562 cells cultured in the two media (10% FBS RPMI and TNB-100) after 2 h of drug exposure, but no differences were observed (data not shown). One can exclude the possibility that the differences in sensitivity observed between the cell lines cultured in serum-free or serum-containing media can be attributed to differences in drug uptake, but it is not possible to exclude a possible effect of differences in cell protein content.

The temperature curves illustrate the substantial differences that exist between the overall lipid profile in 10% FBS RPMI and in TNB-100 cells. Also considered was the activation energy (ΔE), which represents the energy required to dissociate a molecule from the bulk phase. Under our experimental conditions, the cells cultured in TNB-100 medium were significantly more fluid than those cultured in 10% FBS RPMI; however the ΔE values were the same. The opposite pattern between fluorescence polarization and ΔE values in TNB-100 cells could be related to the higher protein concentration. Moreover, the increase in total PL and the decrease in CHOL content, with a consequent increase of the PL/CHOL ratio, is only one but a major factor responsible for the fluidization of cell membranes whereas the increase in protein concentrations may involve a reduction of the fluid phase of the lipids (25).

Extraction of CHOL from the cell membrane by the AL721 lipid mixture (26,27) has been proposed as a mean of enhancing the toxic action of EL. Under this condition, the culture medium composition stays constant, but the CHOL content is reduced (26,27). This approach modified the effect of the drug at 5 μM only at a very early stage of exposure (16,18). The TNB-100 medium may modulate the cytotoxic effects of EL at longer exposure times as well. In fact, the changes produced with the TNB-100 medium reduced the ID_{50} for ET-18-OMe by about 50% in HL60-TNB and K562-TNB cells compared with HL60 and K562 cells at 2, 24 and 48 h exposure times.

We conclude that the composition of the culture medium can modify the cell membrane PL/CHOL ratio and the protein content, thus modulating the biophysical and biochemical effects of EL. The possibility exists that a direct interaction between EL and CHOL or EL and albumin may reduce the availability of the drug. Nevertheless, for the purpose of *ex vivo* bone marrow purging, EL treatment, accompanied by the use of a serum-free TNB-100 medium combined with AL721 treatment, may offer a better therapeutic approach for a more effective elimination of residual myeloid leukemic cells.

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Human Platelets Release a Paf-Acether: Acetylhydrolase Similar to That in Plasma[1]

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Intact washed human platelets aggregated in response to paf-acether (paf) and did not metabolize [^{3}H]paf at concentrations up to 10 nM. However, when platelets were lysed by exposure to pH 9.5, resulting in 37.5 ± 2.5% (mean ± SD, n = 3) lactic dehydrogenase (LDH) release, 20.5 ± 5.7% of the radioactivity was detected as labeled lyso paf and 5.7 ± 3.1% as labeled alkylacylglycerophosphocholine. When platelets were aggregated with 0.5 IU/mL thrombin or high concentrations of paf (100 nM), they released a part of their acetylhydrolase without releasing LDH. In supernatants obtained from aggregated platelets, 21 ± 2% or 10 ± 2% (n = 3), respectively, of the total platelet acetylhydrolase activity was detected *vs.* none in supernatants of resting cells. The release of acetylhydrolase was concentration- and time-dependent and paralleled the release of PF 4, a marker for α-granules. The acetylhydrolase affinity for paf (K_m) measured in sonicates of resting and thrombin-activated platelets was 8.3 ± 1.5 μM *vs.* 10.6 ± 1.5 μM, n = 5, n.s. in a "Mann Whitney" test. The latter K_m was slightly but significantly different ($P < 0.05$, n = 5) from that of the thrombin-released acetylhydrolase (7.9 ± 1.5 μM) and that of the latter was itself different from plasma acetylhydrolase (5.3 ± 0.5, $P < 0.05$, n = 5). Addition of plasma (acid-treated to inactivate acetylhydrolase) decreased the K_m value of supernatant acetylhydrolase to 6.1 ± 1.4 μM. All preparations of acetylhydrolase exhibited similar pH requirements and sensitivity to various inhibitors. Thus paf and thrombin cause release of acetylhydrolase from platelets in parallel with release of the α-granule marker PF4. This phenomenon might represent a protective mechanism against paf-mediated effects in thrombotic and cardiovascular diseases.
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Paf-acether, first described as platelet-activating factor (paf) (2), with the structure of a 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (hexadecyl/octadecyl-acetyl-GPC; 3,4), is a biologically active ether phospholipid which is released from stimulated cells (for reviews, see Refs. 5,6). Paf is hydrolyzed into lyso paf by an acetylhydrolase (6-9), and in platelets an intracellular CoA-independent transacylase reacylates lyso paf to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC, 9,10). Both acetylhydrolase and transacylase metabolize paf in the absence of Ca^{2+} (7,9). The acetylhydrolase is active in a broad pH range (from 6.4 to 8.4) (7) and in plasma it is bound to lipoproteins (11,12).

When incubated under the same conditions as those of paf-induced aggregation, washed intact rabbit platelets metabolize paf (13) but washed intact human platelets do not (14). Many other cell types such as intact neutrophils, activated eosinophils, hepatocytes, macrophages and endothelial cells metabolize exogenous paf (6). Bovine platelets release acetylhydrolase in parallel with serotonin during platelet aggregation in response to paf, adenosine diphosphate and thrombin (15). Murine macrophages when maintained in culture (16) and human monocytes differentiated into macrophages by adherence (17) or incubated with low density lipoprotein (18) acquire the faculty to synthesize and/or to release acetylhydrolase.

In this study, we investigated the release of acetylhydrolase from human platelets. Our results led us to propose that activated platelets might be at least one of the sources of plasma acetylhydrolase.

MATERIALS AND METHODS

Reagents. Aspirin was used as lysine salt Aspegic^R and was obtained from Egic Laboratory (Amilly, France). Fatty acid-free bovine serum albumin (BSA), 5-hydroxytryptamine (serotonin) and β-glucuronidase were purchased from Sigma Chemical Co. (St. Louis, MO). Radiolabeled [^{3}H-*octadecyl*]paf (1-*O*-[^{3}H]octadecyl-2-acetyl-GPC, 80-120 Ci/mmol) and lyso paf (1-*O*-[^{3}H]octadecyl-GPC, 80-120 Ci/mmol) and the scintillation liquids Biodegradable Counting Scintillant (BCS) and Organic Counting Scintillant (OCS) were purchased from Amersham (Bucks, United Kingdom). [^{3}H-*acetyl*]Paf (1-*O*-octadecyl-2-[^{3}H]-acetyl-GPC, 10 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Paf (1-*O*-octadecyl-2-acetyl-GPC), lyso paf (1-*O*-hexadecyl/octadecyl-GPC) and 1-*O*-hexadecyl-2-palmitoyl-GPC were purchased from Bachem (Bubendorf, Switzerland). GF/C filters were obtained from Whatman (Ferriére, France). Human fibrinogen (AB Kabi, Stockholm, Sweden, grade L, pretreated with diisopropyl-fluorophosphate and partially purified was a gift from B.B. Vargaftig, Institute Pasteur, Paris, France). Lactic dehydrogenase (LDH) was purchased from Boehringer (Mannheim, Germany) and platelet factor 4 (PF 4) was obtained from Abbott (Rungis, France). Thrombin was obtained from Hoffman-la-Roche (Basel, Switzerland). Zwittergent^R detergent 3.16 was purchased from Calbiochem (Frankfurt, Germany). Dextrose and citric acid were obtained from Merck (Darmstadt, Germany).

Aggregation of platelets. Washed platelets were prepared according to a method of Korth *et al.* (19). Briefly, human blood was collected in acid citrate dextrose (citric acid, 0.8%, trisodic citrate, 2.2% and 2.5% glucose, 2.45%) in a ratio 1:7 (vol/vol) and platelet rich plasma (PRP) was obtained after centrifugation at 100 × *g* for 15 min. PRP was centrifuged at 900 × *g* for 10 min. The supernatant plasma was centrifuged again to discard remaining platelets and was kept on ice for the acetylhydrolase assay.

[1]This study has been presented in part as a preliminary report at the 72nd Annual Meeting of the Federation of American Societies for Experimental Biology, Las Vegas, NV, May 1988 (1).

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Abbreviations: Alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; BSA, fatty acid-free bovine serum albumin; EC_{50}, 50% maximal aggregation; LDH, lactic dehydrogenase; [^{3}H-*acetyl*]paf, 1-*O*-octadecyl-2-[^{3}H]acetyl-GPC; [^{3}H]paf, 1-*O*-[^{3}H]octadecyl-2-acetyl-GPC; lyso paf, 1-*O*-hexadecyl/octadecyl-GPC; paf, paf-acether, platelet-activating factor, 1-*O*-hexadecyl/octadecyl-2-acetyl-GPC; PF 4, platelet factor 4; PRP, platelet-rich plasma.

Platelets were washed twice in Tyrode's buffer containing (in mM) NaCl, 137; KCl, 2.68; $NaHCO_3$, 11.9; $MgCl_2$, 1.0; NaH_2PO_4, 0.41; dextrose, 0.5; HEPES, 5.0. After 30-min incubation with 0.1 mM aspirin (18), platelets were resuspended at the concentration of 2×10^9 cells per mL and were diluted to a final count of 3×10^8 cells per 300 μL Tyrode's buffer (pH 7.4) in the presence of 1.3 mM $CaCl_2$ and 0.16 mg/mL fibrinogen. Platelets were aggregated with either unlabeled paf (0.1 to 5 nM) or [^{3}H-*octadecyl*]paf (0.65 nM, 15 nCi) for 3 min under stirring at 20 or 37°C. Incubations were also performed in the presence of 0.25% BSA. The extent of platelet aggregation was calculated as percent of maximal light transmission.

*Metabolism of [^{3}-H-*octadecyl]paf in the presence of intact or damaged platelets.* Platelets (5×10^7 cells/500 μL) were incubated with [^{3}H-*octadecyl*]paf (0.65 nM, 15 nCi) without stirring at pH 7.4 for 30 min, 1 h and 2 h either at 20 or 37°C. Incubations were also performed in the presence of BSA. In some experiments, platelets were incubated at pH 9.5 for 30 min at 37°C either in the presence of BSA or in the absence of BSA. Platelet lysis was assessed by the amount of LDH in supernatants (20).

Extraction method and liquid chromatography. After incubation with labeled paf, platelet suspensions or corresponding supernatants were extracted as described (21). Dichloromethane/methanol (1:2, vol/vol) was added to platelet suspensions (0.5 mL) for 24 h at 4°C and then dichloromethane/water (1:1, vol/vol) containing 2% acetic acid was added. Organic phases were collected and water phases were washed three times with 1 vol of dichloromethane. The combined organic phases were brought to dryness under an air stream at 40°C. Dry residues were dissolved in small volumes of mobile phase (dichloromethane/methanol/water, 50:50:5, by vol) and then applied to a Microporasil column 3.9 mm i.d. × 300 mm length (Waters Associates, Milford, MA), which was eluted at a flow rate of 1 mL/min as described (22). Void volume was defined as 3–7 min, 11–13 min as alkylacyl-GPC, 18–21 min as paf and 28–31 min as lyso paf based on appropriate authentic standards. Radioactivity in the fractions was counted using OCS scintillation liquid and was expressed, after subtraction of background values, as percentage of the sum of radioactivity in all fractions. All water phases were also counted to monitor total radioactivity.

Release of acetylhydrolase and granular markers during platelet activation. Platelets were aggregated with thrombin (0.1 to 5 IU per mL) or paf (10 to 500 nM) for 15 s to 5 min. Platelets were centrifuged at 900 × *g* for 10 min at 4°C, and supernatants (5 μg protein) were tested for acetylhydrolase activity as described below. The acetylhydrolase activity in supernatants was expressed as percentage of the total acetylhydrolase activity of sonicated platelets. LDH and PF 4 were measured using commercial kits, and serotonin was measured by an electrochemical method (23) in supernatants and in corresponding pellets.

Acetylhydrolase assay. Acetylhydrolase activity was measured in lysates from resting or thrombin-aggregated platelets, in supernatants of stimulated platelets and in autologous plasma. Platelets were suspended in Tyrod's buffer (pH 6.4) without BSA at a concentration of 10^9 platelets per mL, platelet-free plasma (diluted 1:200, vol/vol in Tyrod's buffer) were added with Zwittergent detergent 3.16 (0.0015% wt/vol, final concentration) and were sonicated (6 pulses of 10 s, at 4°C, Bronson Sonic Power Co., Danbury, CT). Protein content was determined in cell lysates or plasma as described by Lowry *et al.* (24). Lysates from 5×10^7 platelets (50 μL containing 80 μg protein), supernatants from thrombin-activated platelets (50 μL, 5 μg protein) or diluted plasma (1/200, 50 μL, 20 μg protein) were added to 400 μL of a buffer (pH 8.0) containing HEPES 4.2 mM, NaCl 137 mM, KCl 2.6 mM, and EDTA 2 mM. After 5-min preincubation at 37°C, the reaction was initiated by addition of 10 μL [^{3}H-*acetyl*]paf (0.01 μCi, 0.5 to 35 μM final concentration). In some experiments hexadecylpalmitoyl-GPC (20 μM) was added to the assay mixture together with [^{3}H-*acetyl*]paf (10 μM) to determine the specificity of acetylhydrolase toward paf. Preparations containing acetylhydrolase were also treated for 30 min at 37°C with NaF (20 mM) or bromophenacylbromide (0.5 mM) as described by Stafforini *et al.* (25) prior to addition of [^{3}H-*acetyl*]paf for 10 min. In selected experiments, acid-treated plasma that was inactive in our acetylhydrolase assay was added together with platelets' lysate or supernatant obtained from thrombin-activated platelets. Assay mixtures were incubated for 10 min at 37°C unless stated otherwise and stopped by cooling in an ice bath. Two methods were used to separate [^{3}H]acetate from unreacted [^{3}H-*acetyl*]-paf. The first one, described by Palmantier *et al.* (16), was based on the extraction of [^{3}H-*acetyl*]paf in the organic phase by adding 2 mL methanol/dichloromethane (1:1, vol/vol) and 0.4 mL acetate (5%) solution containing [^{14}C]acetate (6000 dpm) as an internal standard. Mixtures were centrifuged at 5000 × *g* for 10 min to facilitate phase separation. The amount of [^{3}H]acetate in the aqueous phase resulting from the acetylhydrolase assay was determined by liquid scintillation counting using BCS as solvent. The second method used to separate [^{3}H]-acetate from unreacted [^{3}H-*acetyl*]paf described by Miwa *et al.* (26), that gave similar results as the former method (data not shown), was based on addition of an excess of BSA (16.7 mg/mL, final concentration) and subsequent precipitation of paf bound to added BSA by trichloroacetic acid (TCA) (7%, vol/vol, final concentration). Denatured proteins were centrifuged at 5000 × *g* for 15 min and the released [^{3}H]acetate was measured by liquid scintillation counting in BCS. Blanks performed using either heat-denatured (100°C for 10 min) or acid-treated (pH 2.0) enzyme were less than 20% of counts. Blanks were subtracted prior to calculations of enzyme activities. Results are expressed in pmol/min/mg protein after correction for extraction losses estimated using [^{14}C]acetate internal standard. The thermolability of acetylhydrolase of enzyme preparations was checked by heating for 30 min at 65°C before addition of [^{3}H-*acetyl*]paf as described (27). The optimal pH of acetylhydrolase using appropriate buffers (from pH 2 to pH 10) was also established.

Statistical analysis. Results are expressed as mean ± SD or SE as indicated. Statistical analysis was performed using the "Mann Whitney" test.

RESULTS

Lack of paf metabolism during platelet aggregation at low concentrations of paf. Paf was not metabolized during

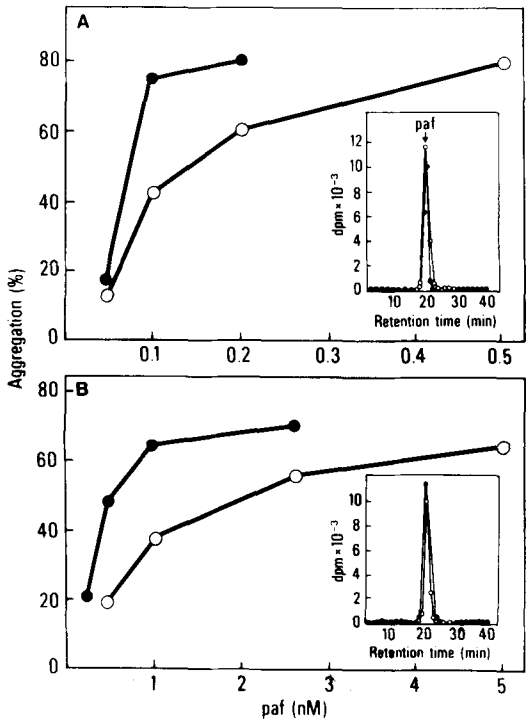

FIG. 1. Concentration-dependent platelet aggregation with paf-acether, platelet-activating factor (paf). Platelets ($3 \times 10^8 \times mL^{-1}$) were incubated with defined concentrations of paf in the absence (A) or in the presence of 0.25% bovine serum albumin (B) at 20°C (●) or 37°C (○) under stirring for 3 min. Values are calculated as percent of maximal light transmission. One experiment is representative of three (statistical analysis is given in the Results section). Insets: Lack of paf metabolism under conditions of platelet aggregation. Platelets were incubated with 15 nCi of 0.65 nM [^{3}H-*octadecyl*]paf at 20°C (●) or 37°C (○) under stirring for 3 min. Phospholipids were analyzed using liquid chromatography as described in Materials and Methods. [^{3}H-*octadecyl*]paf recovered corresponded to 21,279 ± 1369 dpm (mean ± SD, n = 4).

platelet aggregation in response to low concentrations of paf (0.1 to 5 nM) in the absence or presence of BSA and either at 20°C or at 37°C (Figs. 1A and and 1B). The paf concentration inducing 50% of the maximal aggregation (EC_{50}) in the absence of BSA was 0.07 ± 0.02 and 0.1 ± 0.02 nM at 20 and 37°C, respectively (mean ± SD, n = 3). Maximal aggregation was reached after 3 min at 0.2 ± 0.02 and 0.5 ± 0.02 nM paf at 20 and 37°C, respectively (Fig. 1A). BSA (0.25%) inhibited paf-mediated platelet aggregation; EC_{50} values increased to 0.4 ± 0.4 and 0.9 ± 0.2 nM paf after 3-min aggregation with a maximal aggregation at 2.6 ± 0.5 and 5.0 ± 0.5 nM paf at 20 and 27°C, respectively (Fig. 1B).

Stability of [^{3}*H-octadecyl*]*paf in the presence of intact platelets and its catabolism in the presence of damaged platelets.* When intact platelets were incubated with [^{3}H-*octadecyl*]paf (0.65 nM) without stirring either at 20°C (data not shown) or 37°C and either in the presence or in the absence of 0.25% BSA for 30 min, 1 h and 2 h, paf was not degraded (Table 1). Only 4.5% of the label was recovered as [^{3}H]alkylacyl-GPC and 3.5% as [^{3}H]lyso paf at 37°C in the absence of BSA (pH 7.4). Platelets were essentially undamaged since LDH release was less than 9% *vs.* 4.6 ± 0.7% in supernatants of control cells. However, when platelets were partially damaged by incubation at pH 9.5 in the absence of BSA (30 min, 37°C), 5.7 ± 6.2% of the [^{3}H-*octadecyl*]paf was recovered as [^{3}H]alkylacyl-GPC and 20.5 ± 11.4 as [^{3}H]lyso paf (mean ± SD, n = 3 or 4). Under these experimental conditions 14.7 ± 4.9%, 10.5 ± 3.0 and 37.5 ± 2.5% (n = 3) of acetylhydrolase, PF 4 and LDH were released, respectively.

Release of acetylhydrolase during platelet aggregation with thrombin or high concentrations of paf. Platelets released a fraction of their acetylhydrolase content during aggregation in response to thrombin or high concentrations of paf (Table 2). No increase in LDH release was observed after thrombin activation (Table 1) or in response to 100 nM paf (1.5%, n = 2). Unstimulated platelets did not release acetylhydrolase (Tables 2 and 3). The question arose whether acetylhydrolase was released from platelet granules together with one of markers that could help to locate this enzyme within the cell. Activation of platelets by thrombin promoted the release of all types of granule markers: β-glucuronidase (lysosome), serotonin (dense granules) and PF 4 (α-granules), in contrast to paf which only triggered PF 4 release (Table 2). The release of acetylhydrolase and PF 4 in response to thrombin or paf was time-dependent (Table 3). Whereas thrombin (0.5 IU/mL)

TABLE 1

Paf Metabolism by Resting, Damaged or Activated Platelets[a]

Treatment of platelets	LDH (% release)	Lyso paf (%)	Alkylacyl-GPC (%)
(1) 37°C, BSA	4.6 ± 0.7 [3]	0.7 ± 1.2	1.5 ± 1.4 [3]
(2) 37°C	9.0 [2]	3.5 ± 1.2	4.5 ± 3.6 [4]
(3) 30 min, 37°C, BSA, pH 9.5	13.0 [2]	10	0 [1]
(4) 30 min, 37°C, pH 9.5	37.5 ± 4.2 [3]	20.5 ± 11.4	5.7 ± 6.2 [4]
(5) Supernatant of (4)		20.1 ± 1.7	2.7 ± 2.2 [3]
(6) Supernatants of thrombin-activated platelets	6.5 ± 2.6 [3]	29.0	2.2 [2]

[a]Treated or untreated platelets [for (1) to (4) pooled results of 30, 60 and 120 min experiments were used] or their supernatants were incubated with [^{3}H-*octadecyl*]paf (15 nCi, 0.65 nM) for 30 min at 37°C. Labelled phospholipids were extracted, and analyzed as described in Materials and Methods. Results in percentages of the total counts (22,648 ± 1800 dpm) are means ± SD of [n] independent experiments. LDH, lactic dehydrogenase; lyso paf, 1-*O*-hexadecyl/octadecyl-GPC; alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; paf, paf-acether, platelet-activating factor; BSA, bovine serum albumin. Parentheses in Tables 1, 4 and 5 refer to in-table information, not References.

TABLE 2

Acetylhydrolase and Granular Content Release from Stimulated Human Platelets[a]

Stimulation		Acetylhydrolase	β-Glucuronidase	Serotonin	PF 4
Control		0	0	7 ± 3	2
Thrombin	0.2	15 ± 5	3 ± 2	43 ± 28	100
[IU/mL]	0.5	21 ± 3	10 ± 2	71	100
	5	19 ± 5	16 ± 7	80 ± 32	100
Paf	10	0	0	4 ± 2	20
[nM]	100	10 ± 1	0	3 ± 2	22
	500	8 ± 6	0	7 ± 2	27

[a]Platelets (10^8/mL) were incubated for 5 min at 37°C with given agonist and were centrifuged at 900 × *g* for 10 min at 4°C. Supernatants and pellets were tested for enzyme activities, serotonin content and PF 4 content. Results are in percentages of the total (mean ± SD, n = 3 to 5, when SD is omitted n = 2). 100% for acetylhydrolase was 182 pmol/min per mL, for β-glucuronidase 3.8 μg/mL, for serotonin 131 ng/mL, and for PF 4 4.8 μg/mL. Paf, paf-acether, platelet-activating factor; PF 4, platelet factor 4.

TABLE 3

Time-Course of Acetylhydrolase and PF 4 Release from Stimulated Platelets[a]

	Paf (500 nM)		Thrombin (0.5 IU/mL)	
Stimulation (s)	Acetylhydrolase	PF 4	Acetylhydrolase	PF 4
0	1.0 ± 1.7	0.8	2.2 ± 1.6	0.5 ± 0.1
15	8.2	10.2	8.5 ± 1.3	22.0 ± 1.6
30	7.8 ± 4.5	11.8	13.1 ± 6.8	36.1 ± 2.2
60	9.5 ± 6.7	28.5	12.8 ± 8.8	49.2 ± 6.7
120	10.0 ± 2.5	33.6	10.9 ± 5.3	67.9 ± 7.7
180	12.6 ± 6.5	27.5	17.9 ± 4.5	74.9 ± 4.6
300	10.3 ± 2.7	36.1	17.2 ± 5.3	87.7 ± 18.8

[a]Results are expressed as percentages of the total content of acetylhydrolase (mean ± SD, n = 3, when SD is omitted n = 2). For details see footnote to Table 2.

and paf (500 nM) triggered the release of 17.2 ± 5.3% and 10.3 ± 2.7% of the total acetylhydrolase, respectively, PF 4 increased to 87.7 ± 10.8% and 36.1 ± 14.8%, respectively (mean ± SD, n = 3). These data indicate that the releasable pool of the enzyme is limited.

Kinetic studies of acetylhydrolase. The acetylhydrolase activity was measured in plasma (Fig. 2A), in cell lysates (cells and supernatants) from nonactivated and thrombin-activated platelets (Fig. 2B) and in supernatants from thrombin-activated platelets (Fig. 2C). The acetylhydrolase activity in all preparations was linear with the incubation time up at least 10 min and with the protein concentration up to 100 μg/mL (data not shown). Under these conditions [^{3}H]acetate release from [^{3}H-*acetyl*]paf nearly reached a plateau at 20–40 μM (Fig. 2). The acetylhydrolase activity was independent of the presence of Ca^{2+} in the assay (data not shown) and was not inhibited by excess hexadecylpalmitoyl-GPC (Table 4), suggesting that the activity that we measured was not the classical phospholipase A_2. In addition treatment with bromophenacylbromide and NaF similarly affected acetylhydrolase in lysates from thrombin-activated platelets, in corresponding supernatants and in plasma (Table 4). All preparations exhibited similar pH optimum (data not shown).

The K_m values calculated from the Lineweaver-Burk plots are given in Table 5. The statistical analysis of the data showed a small but significant difference of the K_m values when measured in the supernatants as compared to lysates from thrombin-activated platelets and also when compared to autologous plasma enzyme (n = 5, $P < 0.05$). The addition of acid-treated plasma to supernatants decreased the K_m toward the plasma value although the change was not statistically significant. Thrombin stimulation of platelets did not change the affinity of the acetylhydrolase for paf as compared to unstimulated platelets. The V_{max} calculated for acetylhydrolase from Figure 2 are shown in Table 5. These values were calculated in relation to the total protein content of the assay and not to the pure acetylhydrolase protein. Thus they reflect the relative enrichment in acetylhydrolase of the supernatants obtained from the thrombin-stimulated platelets in comparison to the cell lysate.

DISCUSSION

Our data demonstrate the release of acetylhydrolase without release of LDH during platelet aggregation with thrombin or relatively high concentrations of paf. Our results are in good agreement with previous work showing the lack of paf metabolism by human platelets during paf binding (14,19) and/or aggregation (1). Kloprogge and Akkerman (28) found that gel-filtered human platelets may be slightly damaged or activated because they metabolized some of the added paf. In our experiments the presence of BSA either protected platelets against damage during incubation at pH 9.5 or indirectly inhibited acetylhydrolase by binding to the substate (29).

The amount of PF 4, a marker for α-granules, increased in parallel with the acetylhydrolase in supernatants

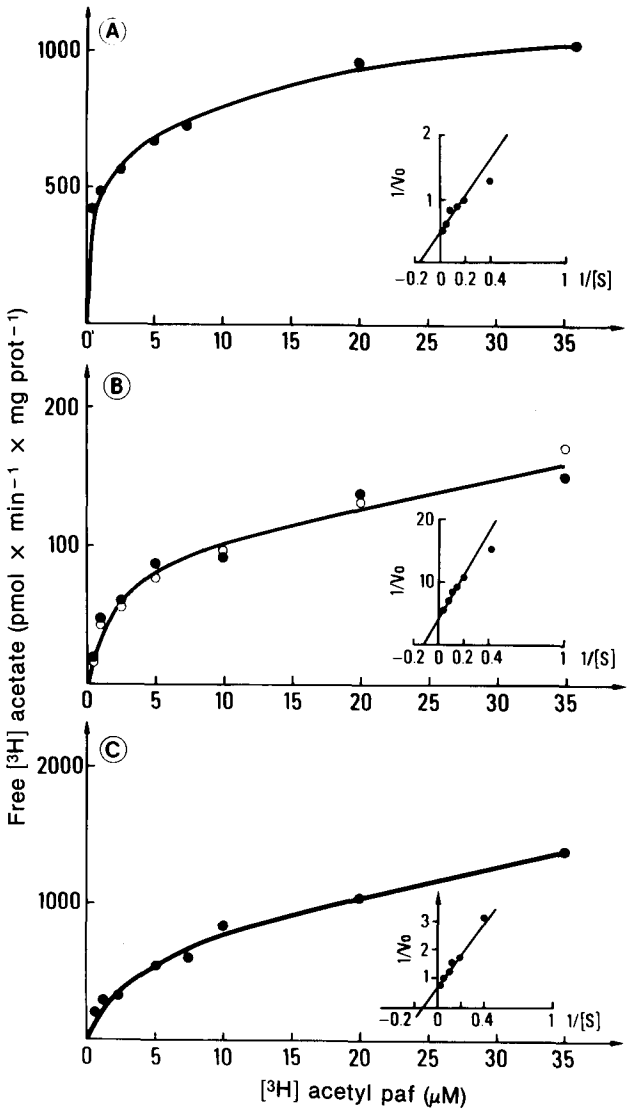

FIG. 2. Acetylhydrolase kinetic constants. The kinetic properties of acetylhydrolase were studied at 37°C for 10 min using 5 to 35 μM of acether, platelet-activating factor (paf) and 0.1 μCi/500 μCi/500 μL of [^{3}H-*acetyl*]paf. The Lineweaver-Burk plots of the data (using manual best fit analysis) are given in insets of each figure A, plasma (20 μg protein/mL); B, lysates (80 μg protein/mL) from resting (●) or thrombin (0.5 IU/mL)-activated (○) platelets; C, supernatants from thrombin-activated platelets (5 μg protein/mL).

of thrombin- and paf-activated platelets whereas it was not released from resting cells. In contrast to stimulation with thrombin, markers for dense granules (serotonin) and lysosomes (β-glucuronidase) were not released when platelets were stimulated with paf. The limited release of acetylhydrolase may either indicate that the major part of this enzyme is present in the cytosolic fraction of platelets or that its releasable pool is limited. It is interesting to note that murine phospholipase A_2 is similarly released from α-granules during platelet activation (30). The release of both hydrolytic enzymes may contribute to the control of the inflammatory process.

The K_m value for paf of acetylhydrolase released from thrombin-activated platelets was slightly but significantly different as compared to the enzyme contained in lyzates from the same cells and in autologous plasma. After addition of acid-treated plasma (inactivation of acetylhydrolase), the K_m of acetylhydrolase in supernatant moved toward that in plasma. Thus the differences in the K_m values for paf, observed for the released enzyme in comparison to that in platelet sonicates and plasma seem to be related to the enzyme protein environment. The treatment of enzyme preparations with various inhibitors indicated that all preparations were only slightly inhibited by NaF, in contrast they were affected up to 50% by bromophenacylbromide. The similarities of pH requirements and the lack of inhibition in the presence of an excess of alkylacyl-GPC let us postulate that platelets among other cells are one of the potential sources of plasmatic acetylhydrolase[2]. Our results are in agreement with those of earlier studies showing similar enzyme kinetics in the plasma (5.7 μM, in ref. 13) and in monocyte/macrophage-like cells (K_m 9.4 ± μM, in ref. 18). Similar results were also obtained using peripheral blood monocytes (17). In addition, after a 24-h culture period, murine peritoneal macrophages rapidly metabolized paf *via* induction of an acetylhydrolase indistinguishable from that in plasma (16).

What might be the biological relevance of the release of acetylhydrolase by platelets in response to paf and thrombin? This release (from platelets as well as, with other agonists, from other cells) may contribute to control the putative paf-mediated effects in (i) hypertensive rats (33), (ii) human Tangier disease (34), (iii) ischemic cerebrovascular diseases and platelet hypersensitivity (35) and (iv) may also be relevant to atherogenesis (36). Acetylhydrolase activity was decreased in rabbit plasma during the late phase of pregnancy in agreement with the important role of paf in enhancing myometrial contractions (27). A familial deficiency of the plasma acetylhydrolase was correlated with respiratory symptoms in asthmatic children (26). In the latter case, the acetylhydrolase level

TABLE 4

Treatment of Preparations Containing Acetylhydrolase with Various Inhibitors[a]

Source of acetylhydrolase	Alkylacyl-GPC (10 μM)	NaF (20 mM)	BPB (0.5 mM)
(1) Thrombin-activated platelets	105 ± 8	84 ± 9	64 ± 10
(2) Supernatant from (1)	115 ± 5	84 ± 8	50 ± 8
(3) Native plasma	115 ± 7	75 ± 3	82 ± 7

[a]Acetylhydrolase activity was measured in lysates of thrombin (0.5 IU/mL, 1 min, 37°C)-activated platelets, in supernatants of the latter, and in native plasma. Samples were preincubated 30 min at 37°C with given inhibitors and incubated further for 10 min at 37°C in the presence of 0.1 μCi [^{3}H-*acetyl*]paf (10 μM). Results in percentages of control (vehicle) are means ± SE of six experiments, except for experiments with alkylacyl-GPC that are means of 4. Values (1 *vs.* 2 *vs.* 3) showed no significant difference in a "Mann Whitney" test. Alkylacyl-GPC, hexadecylpalmitoylglycerophosphocholine; BPB, bromophenacylbromide.

[2]While this manuscript was in the reviewing process Satoh *et al.* (31) and Tarbet *et al.* (32) reported that Hep G2 secrete acetylhydrolase.

TABLE 5

Kinetic Constants of Acetylhydrolase[a]

Source of acetylhydrolase	K_m (μM)	V_{max} (nmol/min/mg protein)
(1) Platelets	8.3 ± 1.5	0.3
(2) Thrombin-activated platelets	10.6 ± 1.5	0.3
(3) Supernatant from (2)	7.9 ± 1.5	1.4
(4) Supernatant from (2) added with acid-treated plasma	6.1 ± 1.4	1.3
(5) Native plasma	5.3 ± 0.5	1.4

[a]Acetylhydrolase activity was measured in lysates of resting platelets or platelets stimulated with thrombin (0.5 IU/mL, 1 min, 37°C), in supernatants of thrombin-stimulated platelets with or without acid-treated (inactivated acetylhydrolase) plasma, and in native plasma. Samples were incubated for 10 min at 37°C in the presence of 0.1 μCi [^{3}H-acetyl]paf and concentrations of unlabeled paf varying from 5 to 35 μM. Results are means ± SD of five separate experiments. Values showed significant differences in "Mann Whitney" test (3 *vs.* 2 and 3 *vs.* 5: $P < 0.05$; 5 *vs.* 1 and 2: $P < 0.003$; n.s.: 2 *vs.* 1, 3 *vs.* 4 and 4 *vs.* 5).

in platelets remained normal although it was deficient in the plasma and the question arose whether the release of acetylhydrolase from platelets of asthmatic children was impaired. Finally, the accumulation of lyso paf was observed in rat alveoli following hypoxia and was attributed to the acetylhydrolase already present in alveolar fluid (37).

In this study we found that washed human platelets do not metabolize added paf. However, when platelets aggregate in response to thrombin or relatively high concentrations of paf, they release (independently of LDH) an acetylhydrolase which is similar to that detected in plasma. Acetylhydrolase may be released from α-granules, a platelet substructure containing other hydrolytic enzymes including phospholipase A_2. These results suggest that activated platelets may participate in the protection against paf-mediated effects in thrombotic and cardiovascular diseases.

ACKNOWLEDGMENTS

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Reaction of α-Tocopherol with Alkyl and Alkylperoxyl Radicals of Methyl Linoleate

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α-Tocopherol was reacted with alkyl and alkylperoxyl radicals at 37°C in bulk phase. The lipid-free radicals were generated by the reaction of methyl linoleate with the free radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) under air-insufficient conditions. The products were isolated by high-performance liquid chromatography. Their structures were identified as 2-(α-tocopheroxy)-2,4-dimethylvaleronitrile (1), a mixture of methyl 9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoate and methyl 13-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoate (2), methyl 9-(α-tocopheroxy)-10(*E*),12(*Z*)-octadecadienoate (3a), methyl 13-(α-tocopheroxy)-9(*Z*),11(*E*)-octadecadienoate (3b), α-tocopherol spirodiene dimer (4) and α-tocopherol trimer (5). When methyl linoleate containing α-tocopherol was oxidized with AMVN under air-sufficient conditions, the main products were 8a-alkylperoxy-α-tocopherones (2). In addition to these compounds, 6-*O*-alkyl-α-tocopherols (1, 3a and 3b) were formed when the reaction was carried out under air-insufficient conditions. The results indicate that α-tocopherol can react with both alkyl and alkylperoxyl radicals during the autoxidation of polyunsaturated lipids.
Lipids 28, 201–206 (1993).

α-Tocopherol, an antioxidant in foods and living cells, inhibits autoxidation of lipids by trapping lipid-peroxyl radicals in two ways (1,2): (i) lipid-peroxyl radicals can be trapped by hydrogen-atom transfer, giving hydroperoxides and α-tocopheroxy radicals; (ii) the resulting α-tocopheroxyl radicals can react with other lipid-peroxyl radicals, or each other, to form nonradical products. The products of the reaction of α-tocopherol with peroxyl radicals have previously been investigated to elucidate the mechanism of autoxidation inhibition by α-tocopherol (3–9).

Alkylperoxyl radicals generated in organic solvents from free radical initiators, such as 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), oxidize α-tocopherol to 8a-alkylperoxy-α-tocopherones and other products (3–8). In our previous study (9), we isolated and characterized the primary products of the reaction of α-tocopherol with peroxyl radicals of methyl linoleate formed in the presence of molecular oxygen and initiated with AMVN. The products were isomeric 8a-(lipid-peroxy)-α-tocopherones. α-Tocopherol also traps alkyl and alkoxyl radicals to give alkylated products (10–13). If lipid peroxidation occurs under oxygen-insufficient conditions, lipid-alkyl radicals can be formed in addition to peroxyl radicals.

In the present study, the reaction products of α-tocopherol during the AMVN-induced oxidation of methyl linoleate under air-insufficient conditions have been isolated and characterized. The possible reaction mechanism of α-tocopherol with free radicals is discussed based on the reaction products observed.

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Abbreviations: AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; MS, mass spectra; UV, ultraviolet.

MATERIALS AND METHODS

Materials. RRR-α-Tocopherol (Type V) was purchased from Sigma Chemical Co. (St. Louis, MO), and *RRR-γ*-tocopherol was prepared from mixed isomers of tocopherol (Hohnen Oil Co., Tokyo, Japan). Tocopherols were purified by Shephadex LH-20 (Pharmacia LKB, Uppsala, Sweden) column chromatography (14). Methyl linoleate (99%, Sigma) was purified and made peroxide-free by silica gel column chromatography (15). The free radical initiator, AMVN, was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification. All solvents were distilled in an all-glass still before use.

High-performance liquid chromatography (HPLC). HPLC was performed using a Jasco Trirotar pump (Japan Spectroscopic Co., Tokyo, Japan) equipped with a Model GP-A40 gradient programmer. A Jasco Model 875-UV detector or a Model 820-FP spectrofluorometer was used as detector. Reversed-phase HPLC was done on a μBondasphere 5μC_8 100Å column (3.9 × 150 mm, Nihon Waters, Ltd., Tokyo, Japan). Preparative reversed-phase HPLC was done using a Wakosil 5C18 column (10 × 300 mm, Wako). Normal-phase HPLC was done on a Wakosil 5Sil column (10 × 300 mm).

Reaction procedure. A mixture of methyl linoleate (10 g, 34 mmol), α-tocopherol (0.20 g, 0.46 mmol) and AMVN (2.0 g, 8.1 mmol) was placed in a beaker (4.5 cm in diameter, specific surface area of 1.3 cm^2/g) and allowed to stand at 37°C for 30 h in the dark. The reaction mixture was concentrated by partitioning between hexane (150 mL) and methanol/water (97.5:2.5, vol/vol; 200 mL) (9). The concentrated products were isolated by preparative reversed-phase HPLC using methanol/ethanol (1:1, vol/vol) at a flow rate of 5.0 mL/min. The trimer in the column was eluted using 2-propanol as the solvent. The products were further purified by normal-phase HPLC using hexane/2-propanol (1000:3, vol/vol) at a flow rate of 4.0 mL/min. The eluent was monitored by measuring absorbance at 260 nm. The compounds isolated were dissolved in ethanol or hexane and stored at −20°C until analysis.

Methyl linoleate (0.20 g or 1.0 g) containing 0.1 or 1 mol% of α-tocopherol and 10 mol% of AMVN was placed in a glass vial (1.4 cm in diameter, specific surface areas of the 0.20-g and 1.0-g samples were 7.7 and 1.5 cm^2/g, respectively) to observe the course of the reaction. At regular intervals, aliquots of the sample were withdrawn, dissolved in ethanol and injected into the HPLC. The reversed-phase column was developed with a 15-min linear gradient of acetonitrile/methanol/water (60:35:5, by vol) to acetonitrile/2-propanol/hexane (35:50:15, by vol) at a flow rate of 1.0 mL/min. The amounts of α-tocopherol were calculated from the peak area by monitoring the fluorescence (ex. 295 nm, em. 340 nm). The amounts of reaction products were calculated from the peak areas by

monitoring elution at 240 nm. γ-Tocopherol was used as the internal standard.

Spectroscopy. ^1H (270.17 MHz) and ^{13}C (67.9 MHz) nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a Jeol JNM-GX-270 FT NMR spectrometer using $CDCl_3$ as solvent and tetramethylsilane as internal standard. Mass spectra (MS) were obtained on a Shimadzu GCMS 9020DF instrument (Shimadzu Co., Kyoto, Japan) operated in the electron impact mode (70 eV). Samples were introduced *via* the direct inlet. Infrared (IR) spectra of samples in liquid film were measured on a Jasco A-302 IR spectrometer. Ultraviolet (UV) spectra were measured with a Jasco Ubest-30 spectrophotometer.

Derivatization. The positions of the double bonds in the reaction products were determined by the oxidative method described by Capella and Zorzut (16). The products were oxidized with osmium tetroxide and the osmates subsequently reduced with sodium sulfite. The hydroxylated products were converted to their corresponding trimethylsilyl ethers with *N,O-bis*(trimethylsilyl)trifluoroacetamide and then analyzed by mass spectrometry.

RESULTS

Characterization of reaction products. AMVN decomposes at 37°C to form alkyl radicals which, in turn, react with molecular oxygen to form alkylperoxyl radicals. The alkylperoxyl radicals attack methyl linoleate to generate the pentadienyl radicals, which react with oxygen to give the methyl linoleate-peroxyl radicals (2). If the supply of oxygen is insufficient, α-tocopherol may react with both the alkyl and the alkylperoxyl radicals.

The reaction products of α-tocopherol with the free radicals were analyzed by reversed-phase HPLC (Fig. 1). Five major peaks, **1, 2, 3, 4** and **5**, that appeared to be the reaction products of α-tocopherol were detected on the chromatogram. When the reaction was carried out under a nitrogen atmosphere, only peaks 1 and 3 in addition to peaks corresponding to methyl linoleate dimer were detected (data not shown). Therefore, peaks 1 and 3 were assumed to be the reaction products of α-tocopherol with

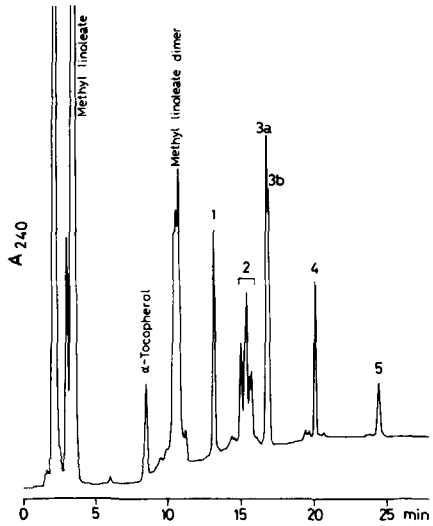

FIG. 1. Reversed-phase high-performance liquid chromatography (HPLC) of the products of the 2,2′-azo*bis*(2,4-dimethylvaleronitrile)-induced reaction of methyl linoleate and α-tocopherol for 30 h. HPLC was done on a μBondasphere 5μC_8 column which was developed with a 15-min linear gradient of acetonitrile/methanol/water (60:35:15, by vol) to acetonitrile/2-propanol/hexane (35:50:15, by vol) at a flow rate of 1.0 mL/min. The eluate was monitored by its absorbance at 240 nm.

alkyl radicals. Peaks corresponding to methyl linoleate dimer were collected by preparative reversed-phase HPLC and obtained as a colorless oil (10.1 mg yield). The structure was identified based on the MS spectrum; *m/z* 555 ($[M - 31]^+$, 1%), 293. (63), 261 (10), 243 (6), 137 (17), 123 (18), 109 (22), 95 (54), 81 (74), and 67 (100). The isomeric structures of the dimer were not investigated. Peaks **1, 2, 3, 4** and **5**, that appeared to be the reaction products of α-tocopherol, were collected by preparative reversed-phase HPLC. Peak **3** was further resolved into two peaks (**3a** and **3b**) by normal-phase HPLC. The structures of **1, 2, 3a, 3b, 4** and **5** were identified as follows (Fig. 2).

Compound **1** was obtained as a colorless oil (27.4 mg yield). Compound **1** was identified as 2-(α-tocopheroxy)-2,4-

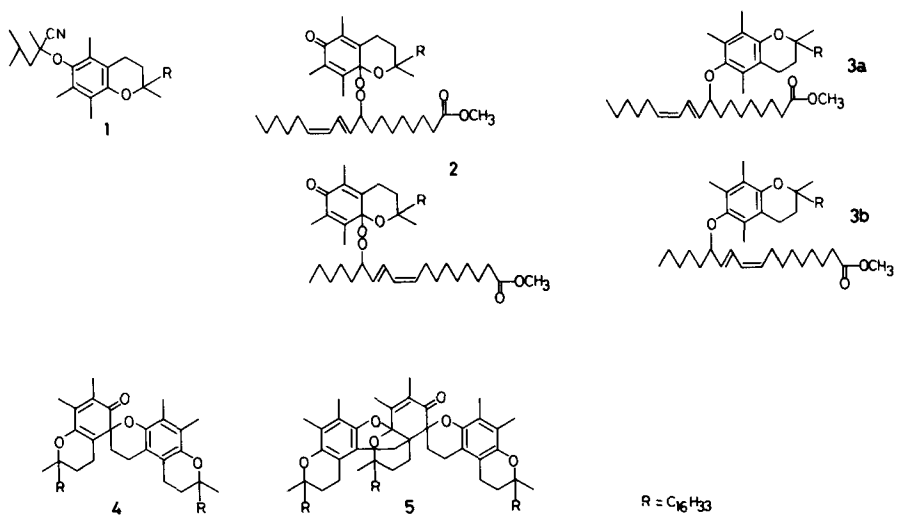

FIG. 2. Structures of compounds 1, 2, 3a, 3b, 4 and 5.

dimethylvaleronitrile: MS *m/z* 513 ([M − CN]$^+$, 6%), 430 (90), 205 (10), and 165 (100); UV (ethanol) λ 228 (ε 11000), 282 (shoulder), and 289 nm (ε 2070); IR (film) ν 2950, 1580, 1460, 1415, 1380, 1250, 1160, 1080, 940, and 920 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.83–0.87 (*m*, 12H), 1.05–2.23 (*m*, 24H), 1.13 (*d*,*J* = 6.8 Hz, 6H), 1.23 (*s*, 3H), 1.46 (*s*, 3H), 1.80 (*m*, 2H), 2.08 (*s*, 3H), 2.19 (*s*, 3H), 2.20 (*s*, 3H), and 2.56 ppm (*dt*,*J* = 6.4 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 12.0, 14.7, 15.6, 19.7, 19.8, 20.8, 21.0, 22.7 (2 atoms), 24.0, 24.1, 24.5, 24.8 (2 atoms), 25.1, 25.6, 28.0, 31.4, 32.7, 32.8, 37.3, 37.5 (3 atoms), 39.4, 40.0, 50.3, 75.0, 76.8, 117.8, 121.5, 123.2, 128.1, 129.8, 143.7, and 148.5 ppm.

Compound 2 was obtained as a mixture (12.6 mg yield) that gave a positive peroxide test (17). The structures were identified by comparison of the HPLC behavior with that of authentic samples as reported previously (9). Compound 2 contained methyl (9*R*,8a*R*)-, (9*R*,8a*S*)-, (9*S*,8a*R*)- and (9*S*,8a*S*)-9-(8a-peroxy-α-tocopherone)-10(*E*),12(*Z*)-octadecadienoates and methyl (13*R*,8a*R*)-, (13*R*,8a*S*)-, (13*S*,8a*R*)- and (13*S*,8a*S*-13)-(8a-peroxy-α-tocopherone)-9(*Z*),11(*E*)-octadecadienoates.

Compounds **3a** and **3b** were obtained as colorless oils (**3a**, 9.3 mg yield; **3b**, 8.9 mg yield). UV spectra of these compounds were identical with absorption maxima at 228 nm (**3a**, ε 30700; **3b**, ε 30500), 282 (shoulder) and 289 nm (**3a**, ε 2500; **3b**, ε 2480) in ethanol. IR spectra of the compounds showed two absorption bands at 950 and 985 cm^{-1} suggesting the presence of *cis-trans* double bonds.

The ^{1}H NMR spectra of the compounds **3a** and **3b** were consistent with those expected for methyl 9-(α-tocopheroxy)-10(*E*),12(*Z*)-octadecadienoate and methyl 13-(α-tocopheroxy)-9(*Z*),11(*E*)-octadecadienoate (Table 1). The geometrical configuration of the conjugated diene system in each compound could be established (18). The *cis-trans* diene systems were present in both compounds, and both *cis* (*J* = 10.3–11.1 Hz) and *trans* (*J* = 14.5–15.4 Hz) coupling constants were observed.

Compounds **3a** and **3b** were oxidized with osmium tetroxide and the osmates were subsequently reduced with sodium sulfite (16). The resulting hydroxylated compounds were silylated and directly analyzed by MS (Fig. 3). The position of the α-tocopheroxy group attached to the fatty acid moiety could be determined from the fragment ion due to α-cleavage of the trimethylsilyloxy group of each derivative (*m/z* 173 for **3a** and *m/z* 259 for **3b**, respectively).

From these results and other spectral data, the structures of **3a** and **3b** were identified as follows. Compound **3a**, methyl 9-(α-tocopheroxy)-10(*E*),12(*Z*)-octadecadienoate: MS *m/z* 430 (64%), 293 (3), and 165 (100); IR (film) ν 2950, 1745, 1460, 1435, 1250, 985, and 950 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 11.9 (2 atoms), 13.1, 14.0, 19.7, 19.8, 20.8, 21.1, 22.5, 22.7 (2 atoms), 23.7, 24.5, 24.8, 25.0, 25.4, 27.7, 28.0, 29.1, 29.2 (2 atoms), 29.6, 31.4, 31.6, 32.8 (2 atoms), 34.1, 35.2, 37.3, 37.5 (2 atoms), 37.6, 39.4, 40.2, 51.4, 74.6, 83.9, 117.3, 122.6, 126.3, 127.9 (2 atoms), 128.4, 132.7, 132.9, 147.0, 147.4, and 174.3 ppm. Compound **3b**, methyl 13-(α-tocopheroxy)-9(*Z*),11(*E*)-octadecadienoate: MS *m/z* 430 (90%), 293 (3), and 165 (100); IR (film) ν 2950, 1745, 1460, 1435, 1250, 985, and 950 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 11.9 (2 atoms), 13.2, 14.1, 19.7, 19.8, 20.8, 21.1, 22.7 (3 atoms), 23.8, 24.5, 24.8, 25.0, 25.2, 27.7, 28.0, 29.0, 29.1 (2 atoms), 29.5, 31.6, 32.0, 32.8, 32.9, 34.1, 35.2, 37.3 (2 atoms), 37.5 (2 atoms), 39.4, 40.1, 51.4, 74.7, 84.0, 117.4, 122.6, 126.4, 127.8, 128.0, 128.4, 132.5, 133.1, 147.1, 147.4, and 174.3 ppm. Compounds **3a** and **3b** proved to be optically inactive each consisting of enantiomeric mixtures.

Compound 4 was obtained as a yellow oil (10.9 mg yield), and compound 5 was obtained as a pale yellow oil (8.4 mg yield). The structures were identified by comparison of the HPLC behavior with those of authentic samples as reported previously (19). Compound 4 was the spirodiene dimer of α-tocopherol and 5 was α-tocopherol trimer.

Reaction products of α-tocopherol in AMVN-induced oxidation of methyl linoleate. Methyl linoleate containing two different concentrations of α-tocopherol was oxidized with AMVN in bulk phase. The reaction was run on two different sample sizes (0.20 and 1.0 g). The exchange of oxygen between air and oil in the 0.20-g sample may be faster than that in the 1.0-g sample because of their different surface areas (the specific surface areas of the 0.20-g and 1.0-g samples were 7.7 and 1.5 cm^2/g, respectively).

TABLE 1

^{1}H NMR Chemical Shifts of 3a and 3b[a]

3a	3b
0.83–0.88 (*m*, 15H)	0.83–0.88 (*m*, 15H)
1.08–2.02 (*m*, 43H)	1.07–2.08 (*m*, 43H)
1.21 (*s*, 3H)	1.21 (*s*, 3H)
2.05 (*s*, 6H)	2.06 (*s*, 6H)
2.11 (*s*, 3H)	2.12 (*s*, 3H)
2.29 (*t*, *J* = 7.7 Hz, 2H)	2.29 (*t*, *J* = 7.7 Hz, 2H)
2.53 (*t*, *J* = 6.4 Hz, 2H)	2.54 (*t*, *J* = 6.4 Hz, 2H)
3.65 (*s*, 3H)	3.65 (*s*, 3H)
4.05 (*dt*, *J* = 7.7, 12.8 Hz, 1H)	4.06 (*dt*, *J* = 7.7, 12.8 Hz, 1H)
5.37 (*dt*, *J* = 7.7, 10.3 Hz, 1H)	5.36 (*dt*, *J* = 7.7, 10.3 Hz, 1H)
5.64 (*dd*, *J* = 8.6, 15.4 Hz, 1H)	5.66 (*dd*, *J* = 7.7, 14.5 Hz, 1H)
5.90 (*dd*, *J* = 10.3, 11.1 Hz, 1H)	5.91 (*t*, *J* = 11.1 Hz, 1H)
6.14 (*dd*, *J* = 11.1, 15.3 Hz, 1H)	6.15 (*dd*, *J* = 11.1, 14.5 Hz, 1H)

[a]Shifts in parts per million downfield relative to tetramethylsilane. Multiplicities are designated by *s*, singlet; *d*, doublet; *t*, triplet; *m*, multiplet. NMR, nuclear magnetic resonance.

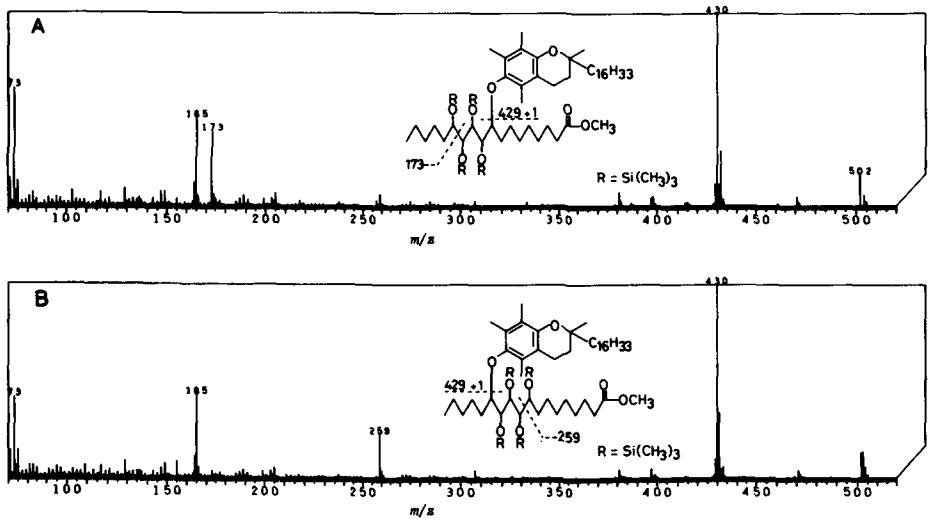

FIG. 3. Mass spectra of trimethylsilyl derivatives of compounds 3a (A) and 3b (B) after hydroxylation of the olefinic bonds by oxidation with OsO_4 and subsequent reduction of the osmates with Na_2SO_3.

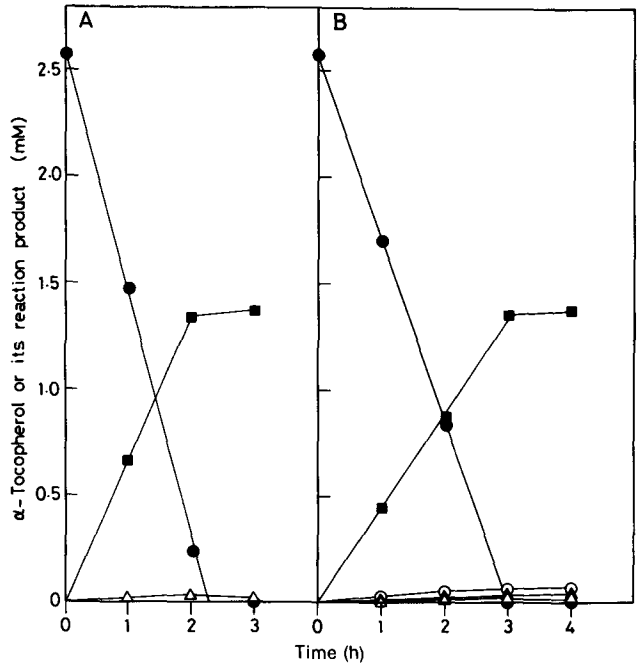

FIG. 4. Reaction of α-tocopherol during the 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (AMVN)-induced oxidation of methyl linoleate containing 0.1 mol% α-tocopherol. Methyl linoleate containing 0.1 mol% α-tocopherol and 10 mol% AMVN was oxidized in two sample sizes in a glass vial; 0.20 g (A) and 1.0 g (B). Residual amounts of α-tocopherol (●) and the reaction products, compound 1 (▲), compound 2 (■), compound 3 (○) and compound 4 (△), in the reaction mixture are shown.

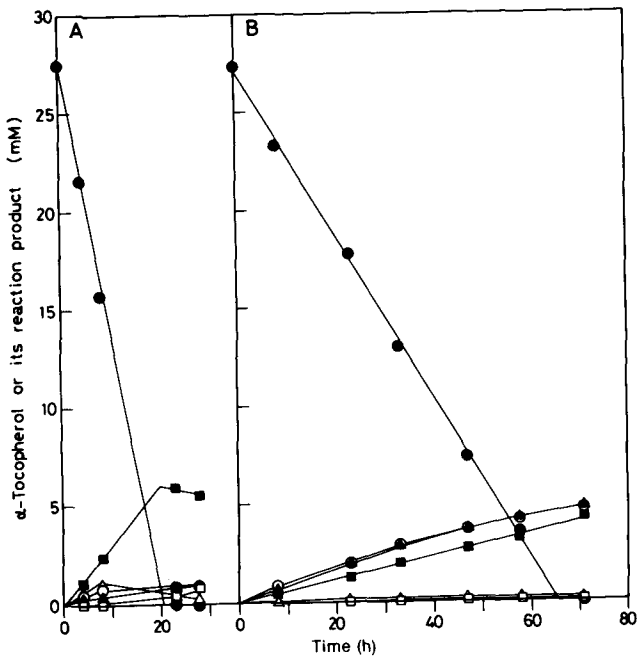

FIG. 5. Reaction of α-tocopherol during the 2,2′-azo*bis*(2,4-dimethylvaleronitrile) (AMVN)-induced oxidation of methyl linoleate containing 1 mol% α-tocopherol. Methyl linoleate containing 1 mol% α-tocopherol and 10 mol% AMVN was oxidized in two sample sizes, 0.20 g (A) and 1.0 g (B). Residual amounts of α-tocopherol (●) and the reaction products, compound 1 (▲), compound 2 (■), compound 3 (○), compound 4 (△) and compound 5 (□), in the reaction mixture are shown.

Figure 4 shows the results of α-tocopherol decay and the formation of the reaction products during the oxidation of methyl linoleate containing 0.1 mol% α-tocopherol. In both sample sizes of 0.20 and 1.0 g, the main reaction products were 8a-alkylperoxy-α-tocopherones (2). Besides a large amount of these compounds, a small amount of 6-*O*-alkyl-α-tocopherols (1, 3a and 3b) was formed in the 1.0-g sample (Fig. 4B).

Figure 5 shows the results of the oxidation of methyl linoleate containing 1 mol% α-tocopherol. The loss of α-tocopherol was affected by sample size. In the 0.20-g sample, α-tocopherol had disappeared by 20 h, and the main products were 8a-alkylperoxy-α-tocopherones (2) in addition to small amounts of 6-*O*-alkyl-α-tocopherols (1, 3a and 3b), dimer (4) and trimer (5) (Fig. 5A). On the other hand, the loss of α-tocopherol was slower in the 1.0-g sample,

and the main products were 8a-alkylperoxy-α-tocopherones (2) and 6-*O*-alkyl-α-tocopherols (1, 3a and 3b) (Fig. 5B). The 1.0-g sample also showed a larger proportion of methyl linoleate dimer; the concentration of methyl linoleate dimer in the reaction mixture after 23-h incubation was 0.84 mM for the 0.20-g sample and 1.71 mM for the 1.0-g sample, respectively.

DISCUSSION

α-Tocopherol is very reactive toward a variety of free radicals and active oxygen species. For example, α-tocopherol traps free radicals generated from certain free radical initiators such as 2,2′-azo*bis*(isobutylnitrile) (20), benzoyl peroxide (21), AMVN (3,6-8) and *tert*-butyl hydroperoxide (4,5,22-24), and it reacts with alkyl and alkoxyl radicals to give alkylated derivatives (10-13). In the present study, α-tocopherol trapped alkyl and alkylperoxyl radicals generated from the AMVN-induced oxidation of methyl linoleate to give 6-*O*-alkyl-α-tocopherols (1, 3a and 3b) and 8a-alkylperoxy-α-tocopherones (2), respectively. The 6-*O*-alkyl-α-tocopherols have been reported to be the reaction products of α-tocopherol with alkyl radicals (10,11,20). We have already reported the isolation and characterization of 8a-alkylperoxy-α-tocopherones as the primary products of α-tocopheroxyl radical with methyl linoleate-peroxyl radicals during autoxidation in bulk phase in the presence of AMVN (9). The dimer (4) and trimer (5) of α-tocopherol were also observed in this study. The dimers and trimers have previously been detected in the autoxidation of methyl linoleate (19).

The oxidation of methyl linoleate (LH) initiated with AMVN (A-N=N-A) and inhibited by α-tocopherol (TOH) in homogeneous solution at sufficient oxygen pressure proceeds by the following reaction sequence (1,2):

Initiation

$$\text{A-N=N-A} \rightarrow 2\text{A}^{\bullet} \quad [1]$$

$$\text{A}^{\bullet} + \text{O}_2 \rightarrow \text{AOO}^{\bullet} \quad [2]$$

$$\text{AOO}^{\bullet} + \text{LH} \rightarrow \text{AOOH} + \text{L}^{\bullet} \quad [3]$$

Propagation

$$\text{L}^{\bullet} + \text{O}_2 \rightarrow \text{LOO}^{\bullet} \quad [4]$$

$$\text{LOO}^{\bullet} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\bullet} \quad [5]$$

Termination

$$2\text{LOO}^{\bullet} \rightarrow \text{stable products} \quad [6]$$

$$\text{LOO}^{\bullet} + \text{TOH} \rightarrow \text{LOOH} + \text{TO}^{\bullet} \quad [7]$$

$$\text{LOO}^{\bullet} + \text{TO}^{\bullet} \rightarrow \text{stable products} \quad [8]$$

$$2\text{TO}^{\bullet} \rightarrow \text{dimer} \quad [9]$$

In the presence of α-tocopherol, Reaction 7 results in the formation of hydroperoxide (LOOH) and the α-tocopheroxyl radical (TO•); the latter reacts rapidly and irreversibly with a second peroxy radical (LOO•) to form stable products (Reaction 8) (25). Our results indicate that the reaction of peroxyl radicals with a low concentration of α-tocopherol under air-sufficient conditions proceeds by Reaction 8 to give the stable products (Fig. 4A). Alternatively, there may be other routes by which some of the tocopheroxyl radicals react, *e.g.*, a bimolecular self-reaction (Reaction 9). Although Reaction 9 is very slow (26,27), dimer and trimer could be formed in the reaction mixture containing a large amount of α-tocopherol (Fig. 5A).

All of the alkyl radicals produced are expected to react very rapidly with oxygen (Reactions 2 and 4); the rate constant of the pentadienyl radical with oxygen is 3.2×10^8 $M^{-1}s^{-1}$ (28). When the oxygen pressure is lowered, the produced alkyl radicals (A• and L•) can react with each other or with α-tocopherol as follows:

$$2\text{A}^{\bullet}(2\text{L}^{\bullet}) \rightarrow \text{dimer} \quad [10]$$

$$\text{A}^{\bullet}(\text{L}^{\bullet}) + \text{TOH} \rightarrow \text{AH(LH)} + \text{TO}^{\bullet} \quad [11]$$

$$\text{A}^{\bullet}(\text{L}^{\bullet}) + \text{TO}^{\bullet} \rightarrow \text{stable products} \quad [12]$$

The alkyl radicals produced from AMVN and methyl linoleate can attack the α-tocopheroxyl radical by Reaction 12 to form 6-*O*-alkyl-α-tocopherols under nitrogen atmosphere. The formation of 6-*O*-alkyl-α-tocopherols was also observed in the larger sample size reaction mixture (Figs. 4B and 5B). Therefore, the supply of oxygen may be dependent on the sample size in the reaction vessel. Reactions 10 and 11 are competing reactions. If Reaction 10 is faster, the α-tocopherol should take longer to be consumed. Our results indicate that the decay of α-tocopherol was lowered and more methyl linoleate dimer was formed under air-insufficient conditions (Fig. 5B). Thus, reactions of alkyl radicals with α-tocopherol may be slower than the reactions of the peroxyl radicals.

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Analysis of Low Erucic Acid Turnip Rapeseed Oil (*Brassica campestris*) by Negative Ion Chemical Ionization Tandem Mass Spectrometry. A Method Giving Information on the Fatty Acid Composition in Positions *sn*-2 and *sn*-1/3 of Triacylglycerols

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A tandem mass spectrometric method is described for the rapid analysis of fatty acid combinations in mixtures of triacylglycerols. Triacylglycerols were introduced into a triple quadrupole mass spectrometer *via* a direct exposure probe and deprotonated using ammonia negative ion chemical ionization. Collisionally activated spectra were obtained and the resulting fragments used to identify the fatty acid constituents, and the fatty acids preferentially located at the *sn*-2 position of the triacylglycerols. Fourteen major molecular weight species of purified triacylglycerols of a supercritical fluid extract of low erucic acid turnip rapeseed oil (*Brassica campestris*) were analyzed. The five major combinations of fatty acids comprised two thirds of the total triacylglycerols and contained oleic, linoleic and α-linolenic acids with linoleic acid favoring the *sn*-2 position.
***Lipids* 28,** 207-215 (1993).

Mass spectrometry has been widely used to analyze the number of carbon atoms and double bonds of constituent fatty acids in natural mixtures of triacylglycerols (TAG). Electron impact-mass spectrometry (EI-MS) produces $M^{+\cdot}$ and $[M-18]^{+\cdot}$ ions, as well as many other characteristic fragment ions, *e.g.*, $[RCO]^+$, $[RCO + 74]^+$, $[RCO + 128]^+$, $[M - RCO_2H]^{+\cdot}$, $[M - RCO_2]^{+\cdot}$ (1,2) and $[RCO + 128 + 14n]^{+\cdot}$ (3). This latter ion series has been used with deuterium labelling of unsaturated linkages (4) in order to determine the position of double bonds in TAG (3). EI-MS has been used to determine the molecular weight distribution of TAG isolated from several edible fats and oils (5).

Murata and Takahashi (6) used positive ion chemical ionization-mass spectrometry (PICI-MS) to analyze several oils, including rapeseed. The intensity of the quasimolecular ion $[M + NH_4]^+$ was shown to be typically 20 times higher than that of the molecular ion in EI-MS. Schulte *et al.* (7) developed improvements for the analysis of solid fats by summing a number of mass spectra during the evaporation of the sample into mass spectrometer. This method corrected for the varying volatilities of the TAG. Field desorption (8) and fast atom bombardment (9) have also been applied to the analysis of TAG, but quantitative information was difficult to obtain and required rigorous calibration.

Various chromatographic interfaces have been used with mass spectrometers in the analysis of TAG mixtures, *e.g.*, gas chromatography (GC)/MS (10-13), reversed-phase liquid chromatography (LC)/CI-MS (14-19) and supercritical fluid chromatography (SFC)/MS (20-22).

In natural fats and oils, all three positions in TAG may be unique, which is of physiological importance. Therefore, in the analysis of TAG it is important to know the locations as well as the combinations of the fatty acids (FA). Ryhage and Stenhagen (1) showed that differentiation between the FA at positions *sn*-2 and *sn*-1/3 was possible in EI-MS by measuring the ions $[M - RCO_2CH_2]^{+\cdot}$. This method has been used in conjunction with on-line capillary SFC/MS (22).

The most common methods used for stereospecific analyses of triacyl-*sn*-glycerols (23) are still based on the principles developed by Brockerhoff (24,25). The methods allow the direct analysis of the fatty acids at positions *sn*-1 and *sn*-2. The fatty acid at position *sn*-3 is then calculated. Several useful modifications of this method have been developed, such as that of Lands *et al.* (26), who replaced the chemical phosphorylation step by specific enzymatic phosphorylation of the 1,2-diacyl-*sn*-glycerols. The methods and their applications have been extensively reviewed by several authors (25-28). There are, however, some difficulties in the stereospecific analyses using enzymes. For example, enzymatic hydrolysis is not always independent of the chain length and number of double bonds (28), and the possibility of acyl migration during the numerous analytical steps must also be taken into account. However, these limitations usually have to be accepted when the goal is to perform a stereospecific analysis of natural fats and oils. Nearly all the analyses over the last quarter of the century have been carried out either by analyzing the original TAG mixtures or simplified mixtures obtained by silver ion chromatography or other fractionation procedures. Only rarely have stereospecific analyses (28,29) been performed on pure "natural" TAG isolated by chromatographic methods. In the stereospecific analyses of TAG mixtures, enantiomer calculations are typically based on various random distribution assumptions. In oil seeds, a "1,3-random-2-random" distribution model has been commonly accepted since the studies of vander Wal (30) and Coleman (31). This distribution model assumes the positions *sn*-1 and *sn*-3 to be identical. However, such distribution models can only be considered as approximations.

Tandem mass spectrometry (MS/MS) is a useful technique for the characterization of individual components in mixtures of analytes. The combination of ammonia negative ion chemical ionization (NICI) and collisional activation (CA) of selected molecular ions is an effective MS/MS method for the analysis of TAG mixtures (32-33). Recently, the research group of Henion (34) has introduced an MS/MS method based on electrospray and $[M + NH_4]^+$ quasimolecular ions. This MS/MS method allowed the fatty acid combinations within a TAG to be identified.

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Abbreviations: ACN, acyl carbon number; CA, collisional activation; DB, number of double bonds; EI-MS, electron impact-mass spectrometry; FA, fatty acid; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; LEAR, low erucic acid rape; MS/MS, tandem mass spectrometry; MW, molecular weight; NICI, negative ion chemical ionization; PICI, positive ion chemical ionization; SFC, supercritical fluid chromatography; TAG, triacylglycerols.

In this paper we describe details and applications of a sensitive ammonia NICI-MS/MS method to analyze TAG. This method allows the combinations of FA within a TAG to be determined and to distinguish between the fatty acids at positions *sn*-2 and *sn*-1/3. The method is applied to the characterization of TAG derived from low erucic acid turnip rapeseed oil (LEAR).

MATERIAL AND METHODS

Turnip rapeseed oil. LEAR (*Brassica campestris*, double zero-variety "Kova") was cultivated in Southwest Finland. Ripe seeds were dried to 5% moisture level and ground on a spice mill with cutting blades. Extraction of the seed oil was performed with chromatography-grade supercritical carbon dioxide at 60°C and 45 MPa (Suprex SFE/50, Suprex Corp., Pittsburgh, PA) (35). The extract was purified by silicic acid chromatography and the lipid fraction dissolved in chloroform (5 mg/mL) and sealed under nitrogen.

Triacylglycerol standards. Reference compounds purchased from Sigma (St. Louis, MO) included: tri-(*all-cis*-5,8,11,14-eicosatetraenoyl)glycerol (20:4-20:4-20:4), 1,2-dioctadecanoyl-3-hexadecanoyl-*rac*-glycerol (18:0-18:0-16:0), 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-3-octadecanoyl-*rac*-glycerol (16:0-18:1-18:0), 1,2-dioctadecanoyl-3-(*cis*-9-octadecenoyl)-*rac*-glycerol (18:0-18:0-18:1), 1,3-dioctadecanoyl-2-(*cis*-9-octadecenoyl)glycerol (18:0-18:1-18:0), 1,2-di-(*cis*-9-octadecenoyl)-3-hexadecanoyl-*rac*-glycerol (18:1-18:1-16:0), 1,3-di-(*cis*-9-octadecenoyl)-2-hexadecanoylglycerol (18:1-16:0-18:1), 1,2-di-(*cis*-9-octadecenoyl)-3-octadecanoyl-*rac*-glycerol (18:1-18:1-18:0) and 1,3-di-(*cis*-9-octadecenoyl)-2-octadecanoylglycerol (18:1-18:0-18:1). Pure TAG fractions (95–100%) from evening primrose oil had combinations 18:3-18:2-18:2, 18:3-18:3-18:2 and 16:0-18:3-18:2. These fractions were analyzed by MS/MS to determine correction factors for 18:2 and 18:3 FA. Correspondingly, the saturated human milk TAG fraction of molecular weight 806.7 consisted mainly of TAG 14:0/16:0/18:0 and 16:0/16:0/16:0, and was used to obtain the correction factor for the 14:0 fatty acid.

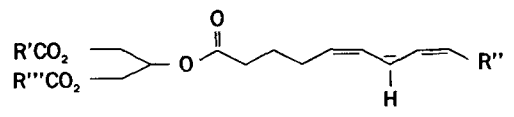
Fatty acid analysis. The fatty acid methyl esters were prepared by transesterification in BF_3/CH_3OH (36,37) and stored in dried hexane at −18°C under nitrogen. The methyl esters were analyzed by capillary gas chromatography/mass spectrometry (35).

Mass spectrometric analysis. Negative ion spectra and tandem mass spectra were obtained on a Finnigan MAT (San Jose, CA) TSQ-70 equipped with a combined EI/CI ion source with removable ion volumes. Negative ions were produced with ammonia CI. Ammonia was introduced into the ion source at an ion source pressure of approximately 8200 millitorr. The ion source temperature was 215°C; electron energy was 70 eV; emission current was 300 μA. Xenon was used as the collision gas in the collision quadrupole, at a pressure of approximately 1.5 millitorr. The offset for the collision quadrupole was 14 eV relative to the ion source. In a typical experiment, the TAG (1 μg) dissolved in dichloromethane (1 μL) was placed at the end of the standard direct exposure probe, the solvent was evaporated and the probe was introduced directly into the ion source. The analysis was started and the temperature of the probe was increased to 310°C at a rate of 300°C/min (the maximum heating rate of the probe). Daughter ion spectra were obtained by selecting the $[M - H]^-$ molecular ions with the first quadrupole mass spectrometer, collisionally activating these ions in the second quadrupole and scanning the third quadrupole from 50 amu to just above the $[M - H]^-$ ion. Spectra were acquired over a period of three minutes with a scan time of eight seconds. The first and last spectra were ignored and the remaining spectra were averaged and displayed. When the relative proportions of the $[M - H]^-$ ions were used for quantitative comparisons, corrections were made for the ^{13}C isotope content of the ions.

Combinations of FA in TAG. The type and ratio of FA within each molecular weight fraction of TAG were determined by obtaining daughter ion spectra of the corresponding $[M - H]^-$ ions. However, in order to analyze the proportions of the fatty acids within each molecular weight group, molar correction factors based on the abundances of the RCO_2^- ions were required. The correction factors for fatty acids were determined from the intensities of RCO_2^- ions of TAG standards. The factor 1.0 was given to octadecanoic acid and the other correction factors determined were: 14:0 = 1.3, 16:0 = 1.1, 16:1 = 1.5, 18:1 = 1.3, 18:2 = 1.6 and 18:3 = 1.9.

RESULTS AND DISCUSSION

MS analysis of reference compounds. Ammonia under chemical ionization conditions produces the reagent ion H_2N^- which is a powerful gas phase base (38,39) capable of deprotonating most organic substrates, including ketones and esters. TAG are deprotonated on the carbon α to the carbonyl group. Figure 1 and Table 1 list the partial, CA daughter spectra of $[M - H]^-$ ions derived from standard TAG. The most obvious feature of these spectra are their simplicity, with only four distinct groups of ions dominating the spectra, *i.e.*, $[M - H{-}RCO_2H]^-$ (very low intensity), $[M - H{-}RCO_2H{-}74]^-$, $[M - H{-}RCO_2H{-}100]^-$ and RCO_2^-. An exception was the spectrum of the dodeca-unsaturated tri-(all-*cis*-5,8,11,14-eicosatetraenoyl)-glycerol (triarachidonoylglycerol), which showed a number of additional fragmentations (Fig. 1c). This increase in fragmentations of triarachidonoylglycerol may be explained by the formation of a competing $[M - H]^-$ molecular ion. The competing ion is formed by deprotonation α to one of the double bonds on a fatty acid substituent, such as *a*.



a

CA of this ion results in additional fragmentations and increased complexity in the spectra of highly unsaturated TAG. Fortunately, in many edible fats and oils, most of these additional fragmentation processes are minor and do not seriously interfere with the sensitivity or the specificity of the technique.

Examination of the data presented in Table 1 reveals that the production of $[M - H{-}RCO_2H]^-$, $[M - H1{-}RCO_2H{-}74]^-$ and $[M - H{-}RCO_2{-}100]^-$ occurs primarily with the loss of the fatty acids from position *sn*-1/3.

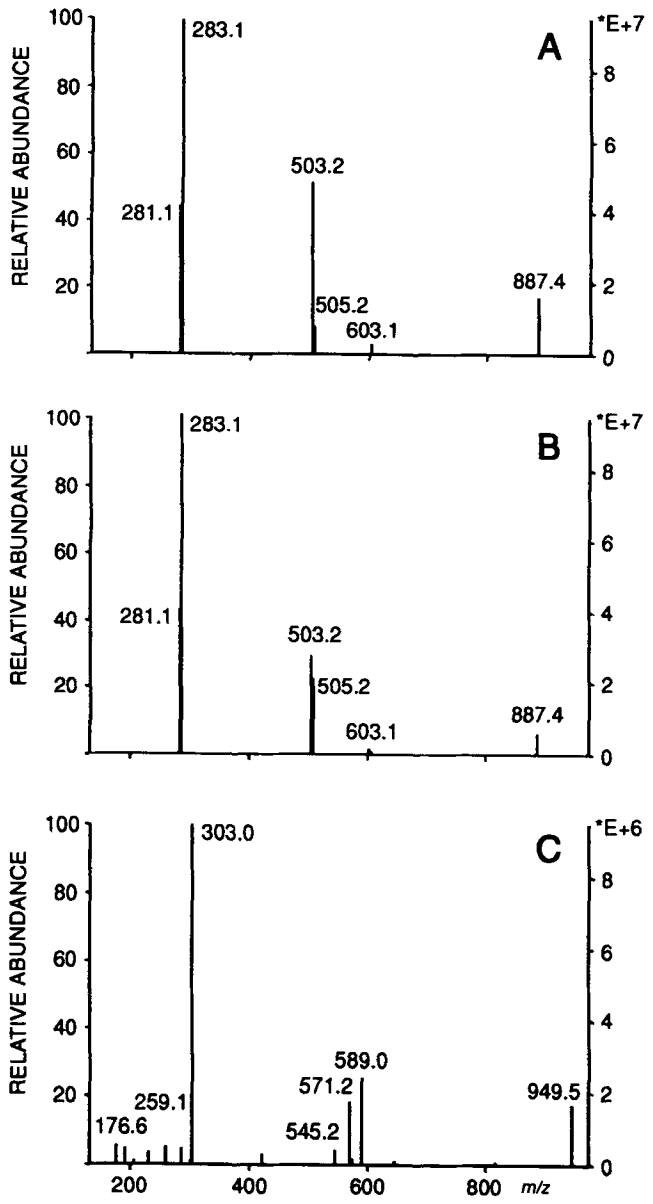


FIG. 1. Collisionally activated daughter spectra of the $[M-H]^-$ parent ions of A, 1,3-distearoyl-2-oleoylglycerol (18:0-18:1-18:0); B, 1,2-distearoyl-3-oleoyl-*rac*-glycerol (18:0-18:0-18:1); and C, triarachidonoylglycerol (20:4-20:4-20:4).

Again, as an exception, the $[M-H-RCO_2H-74]^-$ ion, which is a minor fragmentation in most spectra, is a major fragmentation in the spectrum of triarachidonoylglycerol.

The intensities of the RCO_2^- fragment ions also depend strongly on the composition of the TAG. Altogether, there are three compositional factors that affect the formation of RCO_2^-, namely (i) the number of double bonds on the FA substituent, (ii) the FA chain length and (iii) the regiospecific position of the fatty acid substituent. The formation of RCO_2^- seems to occur through a number of fragmentation mechanisms occurring at different rates. Some of these fragmentation processes are specific to the *sn*-1/3 carbons. This can be seen when the abundance of the RCO_2^- ions from the MS/MS spectrum of 1-palmi-

TABLE 1

Ammonia Negative Ion Chemical Ionization Collisional Activated Daughter Spectraa of Some Reference Triacylglycerols (TAG)

TAG[a]	Negative ion species								
$FA_1/FA_2/FA_3$	$[M-H]^-$	$[FA_1-H]^-$	$[FA_2-H]^-$	$[FA_3-H]^-$	$[M-H-FA_1]^-$	$[M-H-FA_2]^-$	$[M-H-FA_3]^-$		
18:1-16:0-18:1	78.0	100.0	84.0	100.0	2.3	0.5	2.3		
18:1-18:1-16:0	16.4	100.0	100.0	52.5	0.6	0.6	0.7		
16:0-18:1-18:0	4.8	93.5	80.7	100.0	0.9	0.3	1.2		
18:0-18:0-16:0	45.0	100.0	100.0	41.7	1.0	1.0	0.7		
18:1-18:1-18:0	5.4	100.0	100.0	59.4	1.5	1.5	1.3		
18:1-18:0-18:1	33.0	100.0	58.6	100.0	1.2	0.4	1.2		
18:0-18:1-18:0	16.8	100.0	44.8	100.0	2.7	0.5	2.7		
18:0-18:0-18:1	6.7	100.0	100.0	43.0	1.5	1.5	1.0		
20:4-20:4-20:4	8.9	100.0	100.0	100.0	0.9	0.9	0.9		
	$[M-H-FA_1-100]^-$		$[M-H-FA_2-100]^-$		$[M-H-FA_3-100]^-$	$[M-H-FA_1-74]^-$	$[M-H-FA_2-74]^-$	$[M-H-FA_3-74]^-$	
18:1-16:0-18:1	50.2		6.5		50.2	0.4	0.1	0.4	
18:1-18:1-16:0	20.3		20.3		15.9	0.7	0.7	0.4	
16:0-18:1-18:0	34.3		8.9		37.3	1.6	0.2	1.6	
18:0-18:0-16:0	17.8		17.8		12.3	0.2	0.2	0.3	
18:1-18:1-18:0	32.3		32.3		25.8	0.7	0.7	0.6	
18:1-18:0-18:1	26.0		3.8		26.0	0.4	0.1	0.4	
18:0-18:1-18:0	51.9		8.3		51.9	1.1	0.2	1.1	
18:0-18:0-18:1	29.1		29.1		22.4	0.7	0.7	0.4	
20:4-20:4-20:4	2.8		2.4		2.4	9.5	9.5	9.5	

[a]FA, fatty acids. FA_2 is at the position *sn*-2; FA_1 and FA_3 randomly occupy the positions *sn*-1 and *sn*-3.

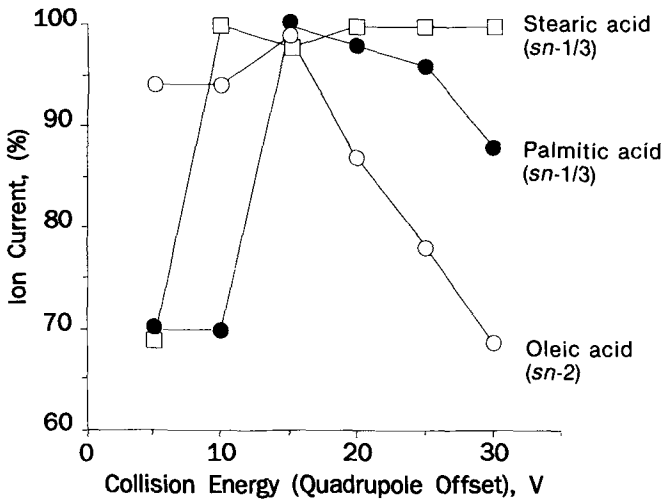


FIG. 2. Plot of RCO_2^- abundances from negative ion chemical ionization collisionally activated daughter spectra of 1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol, showing variation in abundances with different collision energies.

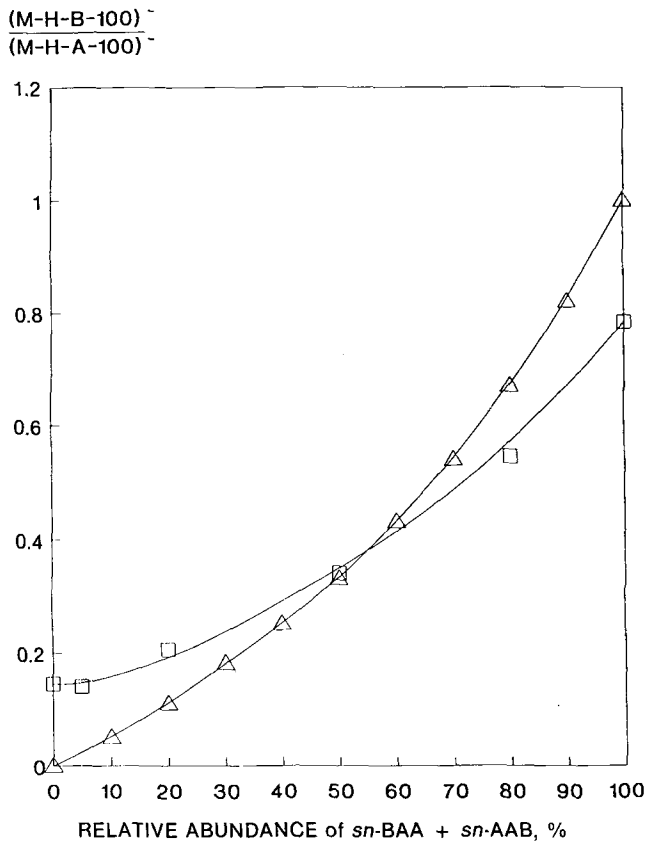


FIG. 3. Ratio of the ions $[M - H - B - 100]^-/[M - H - A - 100]^-$ in daughter spectra of binary mixtures of triacylglycerols of type A-A-B and A-B-A. A and B represent different fatty acids at regiospecific locations, *i.e.*, *sn*-1/3 and *sn*-2. Measurements were based on the intensities of the $[M - H - RCO_2H - 100]^-$ fragment ions. The squares represent the empirical correlation determined using mixtures of known ratios of reference compounds listed in Table 1. The triangles show the theoretical curve if no $[M - H - RCO_2H - 100]^-$ ions concerning the fatty acid at position *sn*-2 were formed.

toyl-2-oleoyl-3-stearoyl-*rac*-glycerol are plotted at different collision energy (Fig. 2). The results in Figure 2 indicate that the formation of RCO_2^- from position *sn*-2 has different energy requirements than the formation of RCO_2^- from position *sn*-1/3. Therefore, some or all of the RCO_2^- ions from the *sn*-2 position must be formed by a different fragmentation mechanism. In order to minimize this effect, it is essential to hold the collision energy at a fixed value that preferably does not discriminate against the *sn*-2 fragment. The other factor affecting the formation of RCO_2^- is the number of double bonds and the chain length. MS/MS results from racemic reference TAG compounds, and other TAG mixtures show that RCO_2^- ions with double bonds are consistently underestimated. The discrimination between unsaturated FA and FA of different chain lengths may be corrected by obtaining correction factors from reference TAG. It is also important to obtain the correction factors at the same collision energy with which the oil samples will be analyzed.

Information about regiospecific positions of fatty acids. The data in Table 1 and Figures 1a and 1b show that the formation of the ions $[M - H-RCO_2H]^-$, $[M - H-RCO_2H-74]^-$, and $[M - H-RCO_2H-100]^-$ in the MS/MS spectra of TAG occurs in from little to none of the *sn*-2 fatty acid. These ions can therefore be used to determine the fatty acid located at *sn*-2 position. It is convenient to use the $[M - H-RCO_2H-100]^-$ ion for this purpose, as it is the most abundant fragment, thus increasing the sensitivity of the technique.

In a simple, two FA system, the mixture of TAG may be considered to consist of TAG *rac*-A-A-B and A-B-A. A and B in these systems represent FA at different regiospecific locations; *e.g.*, in the system A-B-A, fatty acid B is at the *sn*-2 position. In order to determine the possibility of accurately calculating the regiospecificity in a TAG system of this type, the ratio of the MS/MS fragment ions $[M - H-B-100]^-/[M - H-A-100]^-$ from mixtures of TAG of the type A-A-B and A-B-A were compared. It was found in pure TAG systems of A-A-B and A-B-A that the ratio of the intensities $[M - H-B-100]^-/[M - H-A-100]^-$ were (A-A-B) 0.784 ± 0.015 and (A-B-A) 0.145 ± 0.016, respectively. These ratios were obtained regardless of the fatty acid combinations (Table 1). The different combinations of the binary mixture A-A-B and A-B-A showed an increase in the ratio of $[M - H-B-100]^-/[M - H-A-100]^-$ in going from 100% A-B-A to 100% A-A-B, as seen in Figure 3. This result indicates that in simple mixtures it is possible to determine not only whether the mixture consists of different regioisomeric TAG, but also the ratio of the isomers. There are a number of possibilities why some of the $[M - H-RCO_2H-100]^-$ is formed from the fatty acid at *sn*-2 position. These are: (i) rearrangement of the samples *via* acyl migration occurred during volatilization into the MS; (ii) the reference compounds were not pure isomers or; (iii) a small amount of $[M - H-RCO_2-100]^-$ is produced from the *sn*-2 fatty acid. Although we were unable to discount any one of these possibilities, experiments with different probe heating rates suggested that some acyl migration occurs during volatilization.

Analysis of turnip rapeseed oil. The fifteen major FA from the purified turnip rapeseed oil TAG analyzed as methyl esters by GC/MS are listed in Table 2. Only seven of the fatty acids exceeded 1% of the total fatty acids in

TABLE 2

Proportions of the Major Fatty Acids of Triacylglycerols (TAG) in the Turnip Rapeseed Oil, Variety "Kova"

Peak number (GC)	Fatty acid	Relative abundance, mol%	
		GC/MS	MS/MS[a]
1	14:0	0.1	trace
2	16:0	3.4	5.0
3	16:1n-7	0.2	0.6
	16:2	—	trace
4	18:0	1.5	3.5
5	18:1n-9	54.9	50.0
6	18:1n-7	2.7	
7	18:2n-6	22.1	27.8
8	18:3n-3	12.7	12.2
9	20:0	0.4	0.1
10	20:1n-9	1.0	0.4
11	20:2n-6	0.1	trace
12	20:3n-3	0.2	—
13	22:0	0.2	trace
14	22:1n-9	0.3	trace
15	24:0	0.2	—

[a]Represents TAG of the fourteen molecular weight species analyzed. Corrected due to ^{13}C species in NICI-MS and due to varying chain lengths and number of double bonds of the fatty acids in the collisionally activated daughter spectra. Abbreviations: GC, gas chromatography; NICI-MS, negative ion chemical ionization-mass spectrometry.

the TAG mixture. The most abundant were *cis*-9-octadecenoic (oleic, 18:1n-9), *cis,cis*-9,12-octadecadienoic (linoleic, 18:2n-6) and all-*cis*-9,12,15-octadecatrienoic (α-linolenic, 18:3n-3) acids. It is noteworthy that the content of *cis*-13-docosenoic (erucic, 22:1n-9) acid was below 1%. The major saturated fatty acids were palmitic (16:0), stearic (18:0) and eicosanoic (20:0) acids. Characterization of turnip rapeseed oil fatty acids, the acyl carbon number profile and the molecular weight distribution of TAG from the variety "Kova" during the growing period and maturation have been reported elsewhere (35). The major fatty acids in "Kova" have been shown to be practically identical with the LEAR cultivars "Janpol" (40) and "Candle" (41). The cultivar "Tower" contains more oleic (64%) and less erucic acid (0.08%) than the other varieties (41). Details of the minor fatty acids in the low erucic varieties "Span" and "Tower" have been extensively studied by Sebedio and Ackman (42).

An NICI mass spectrum of the TAG from turnip rapeseed oil ("Kova") is shown in Figure 4. The five clusters in the spectrum correspond to $[M - H]^-$ ions of ACN 50, 52, 54, 56 and 58. The major clusters were ACN 54 and ACN 52 and comprised 76 and 18% of the total ion count, respectively. Daughter spectra were obtained from the fourteen $[M - H]^-$ ions numbered in Figure 4. Results of the mass spectrometry analyses of turnip rapeseed oil are listed in Table 3.

The proportions of the MW fractions (878.7–884.7 Da) of ACN 54 were consistent with earlier results derived from ammonia PICI-MS (35). The deviations between the PICI results and the NICI results may occur as a result of the PICI data being corrected for ^{13}C isotope content, fatty acid chain lengths and the number of double bonds. In contrast, the NICI data were only corrected for the ^{13}C isotope content. The ratios of FA within an MW fraction were calculated directly from the abundances of the RCO_2^- ions in the daughter spectra. Quantitative correction factors were then applied to the RCO_2^- ion abundances to correct for the discrimination in the detection of the different FA (TAble 3). The total FA profile of the TAG analyzed by both GC/MS as methyl esters and directly by MS/MS is shown in Table 2. Only the most abundant MW species of the minor ACN clusters were ana-

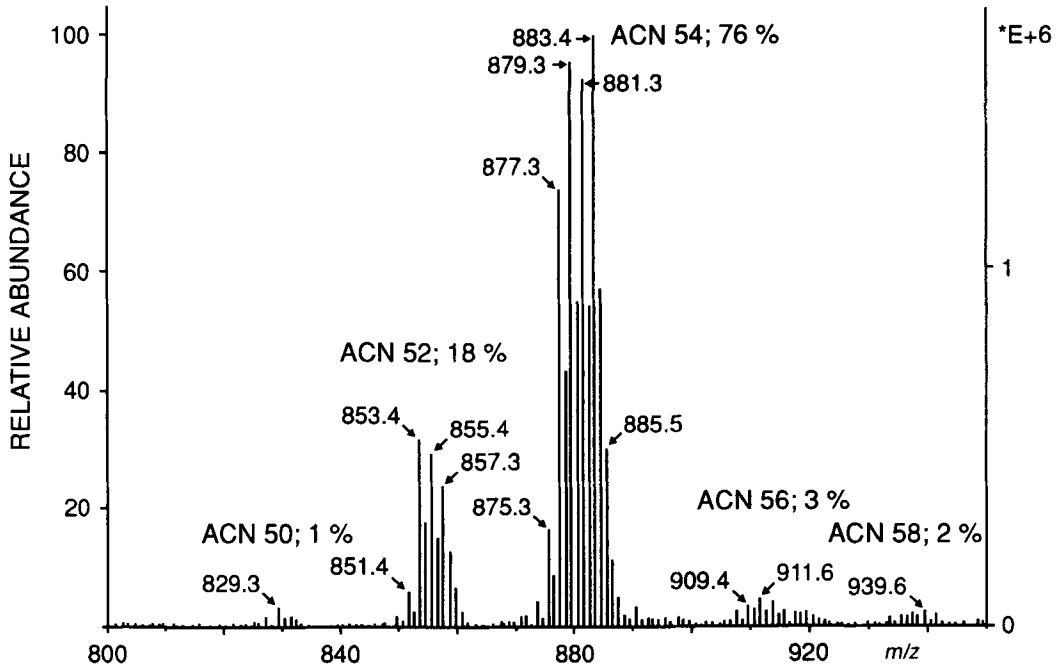


FIG. 4. Ammonia negative ion chemical ionization mass spectrum of purified triacylglycerol fraction from a supercritical fluid extract of turnip rapeseed oil, variety "Kova".

TABLE 3

Ammonia Chemical Ionization Tandem Mass Spectra Results of Purified Triacylglycerols of Turnip Rapeseed Oil Obtained by Supercritical Fluid Extraction

Triacylglycerols								Fatty acids				Loss of (FA−100)		
				Rel. abund.						Rel. Ab.	Proport.			Rel. Abund.
ACN	MW	DB	$[M-H]^-$	$[M-H]^{-a}$	$[M+18]^{+b}$	Combination of FA[c]	Proportion of TAG, %	$[FA-H]^-$	FA	$[FA-H]^-$	of FA[d]	$[M-H-FA-100]^-$	FA	$[M-H-FA-100]^-$
50														
	830.7	2	829.7	0.8	—	16:0/16:0/18:2[e]	62	227	14:0	3.8	3.9	501	14:0	6.4
						16:1/16:0/18:1	24	253	16:1	5.4	6.5	475	16:1	7.1
						14:0/18:1/18:1	14	255	16:0	55.0	48.6	473	16:0	52.4
								279	18:2	14.9	19.1	449	18:2	10.2
								281	18:1	20.9	21.8	447	18:1	23.7
52														
	852.7	5	851.7	1.4	—	16:0/18:3/18:2[e]	79	251	16:2	1.7	1.3	499	16:2	1.8
						16:1/18:3/18:1	16	253	16:1	5.4	4.0	497	16:1	10.4
						16:2/18:2/18:1	5	255	16:0	26.9	20.2	495	16:0	30.8
						16:1/18:2/18:2	trace	277	18:3	23.9	31.1	473	18:3	25.6
								279	18:2	29.9	32.6	471	18:2	18.4
								281	18:1	12.2	10.9	469	18:1	13.0
	854.7	4	853.7	6.8	2	16:0/18:3/18:1	51	253	16:1	4.4	4.8	499	16:1	6.5
						16:0/18:2/18:2	34	255	16:0	33.6	26.7	497	16:0	29.2
						16:1/18:2/18:1	15	277	18:3	11.6	15.9	475	18:3	12.2
								279	18:2	25.5	29.4	473	18:2	28.9
								281	18:1	24.9	23.3	471	18:1	23.3
	856.7	3	855.7	5.5	2	16:0/18:2[e]/18:1	77	253	16:1	3.4	3.8	501	16:1	2.5
						16:1/18:1/18:1	14	255	16:0	27.6	22.6	499	16:0	34.3
						16:0/18:3/18:0	9	277	18:3	2.5	3.6	477	18:3	0.8
						16:1/18:2/18:0	trace	279	18:2	29.1	34.6	475	18:2	21.9
								281	18:1	34.3	33.2	473	18:1	36.8
								283	18:0	3.0	2.3	471	18:0	3.7
	858.7	2	857.7	4.5	2.4	16:0/18:1/18:1[e]	91	255	16:0	33.0	29.3	501	16:0	32.2
						16:0/18:2/18:0	9	279	18:2	5.3	6.8	477	18:2	9.3
								281	18:1	59.1	61.9	475	18:1	51.5
								283	18:0	2.5	2.0	473	18:0	6.9
54														
	876.7	7	875.7	3.7	13.4	18:3/18:3/18:1	57	277	18:3	45.2	51.7	497	18:3	60.1
						18:3/18:2/18:2	43	279	18:2	30.5	29.3	495	18:2	24.6
								281	18:1	24.3	19.0	493	18:1	15.2
	878.7	6	877.7	15.6	15.0	18:3/18:2[e]/18:1	81	277	18:3	25.6	30.6	499	18:3	36.1
						18:2/18:2/18:2	19	279	18:2	45.0	45.3	497	18:2	31.0
								281	18:1	29.4	24.1	495	18:1	32.9
	880.7	5	879.7	18.8	21.1	18:2/18:2[e]/18:1	50	277	18:3	13.3	17.2	501	18:3	18.2
						18:3/18:1/18:1	47	279	18:2	32.7	35.5	499	18:2	27.5
						18:3/18:2/18:0	2	281	18:1	52.8	46.5	497	18:1	51.9
								283	18:0	1.2	0.8	495	18:0	2.5

	882.7	4	881.7	17.8	17.5	18:2/18:1/18:1	89	277	18:3	2.0	2.7	503	18:3	6.1
						18:3/18:1/18:0	7	279	18:2	32.2	36.9	501	18:2	26.4
						18:2/18:2/18:0	4	281	18:1	61.3	57.1	499	18:1	64.2
								283	18:0	4.5	3.2	497	18:0	3.4
	884.7	3	883.7	19.3	17.8	18:1/18:1/18:1	82	279	18:2	4.9	6.0	503	18:2	7.8
						18:2/18:1/18:0	18	281	18:1	87.0	87.7	501	18:1	86.7
								283	18:0	8.1	6.3	499	18:0	5.5
	886.7	2	885.7	3.7	1.7	18:1/18:1/18:0[e]	94	279	18:2	1.4	1.9	505	18:2	6.2
						18:2/18:0/18:0	6	281	18:1	57.6	63.5	503	18:1	64.7
								283	18:0	40.9	34.7	501	18:0	29.0
56														
	910.8	4	909.8	0.8	1.0	18:2[e]/18:1/20:1	69	277	18:3	3.5	5.2	531	18:3	3.4
						18:3/18:1/20:0	14	279	18:2	29.0	35.8	529	18:2	17.0
						18:2/18:2/20:0	14	281	18:1	29.3	29.4	527	18:1	35.8
						18:1/18:1/20:2	3	307	20:2	1.3	1.0	501	20:2	5.3
								309	20:1	26.4	20.4	499	20:1	32.9
								311	20:0	10.6	8.2	497	20:0	5.5
	912.8	3	911.8	0.9	1.0	18:1/18:1/20:1	76	279	18:2	8.0	10.5	531	18:2	4.3
						18:2/18:0/20:1	24	281	18:1	57.5	61.2	529	18:1	49.5
								283	18:0	6.7	5.5	527	18:0	3.6
								309	20:1	27.9	22.8	501	20:1	42.6
58														
	940.8	3	939.8	0.5	—	18:1/18:1/22:1	49	255	16:0	1.5	1.4	583	16:0	2.7
						18:2/18:1/22:0	39	277	18:3	1.0	1.6	561	18:3	1.0
						16:0/18:2/24:1	6	279	18:2	12.3	16.6	559	18:2	12.1
						18:3/18:0/22:0	3	281	18:1	33.2	36.4	557	18:1	34.0
						18:2/20:0/20:1	3	283	18:0	1.2	1.0	555	18:0	2.0
						18:1/20:1/20:1	1	309	20:1	5.1	4.3	529	20:1	6.5
						18:2/18:0/22:1	trace	311	20:0	0.7	0.6	527	20:0	1.5
						18:3/20:0/20:0	trace	337	22:1	22.6	19.1	501	22:1	19.6
								339	22:0	20.0	16.9	499	22:0	15.6
								365	24.1	2.5	2.1	473	24:1	5.2

[a]Ratios of the fourteen MW fractions of triacylglycerols (TAG) analyzed by NICI-MS. The values have been corrected for ^{13}C content.
[b]Ratios of the MW fractions of TAG analyzed by PICI-MS, values are corrected for ^{13}C content, the chain length and degree of unsaturation of FA (ref. 33).
[c]The slashes between FA do not indicate the regiospecific positions within the TAG.
[d]Proportions of FA were calculated by taking relative abundances of the $[FA-H]^-$ and multiplying by correction factors.
[e]FA preferentially (>67%) located at the *sn*-2 position.

Abbreviations as in Table 2 and MW, molecular weight; DB, double bond; PICI, positive ion chemical ionization; FA, fatty acid; ACN, acyl carbon number.

lyzed by MS/MS, whereas the GC/MS analysis represents the total oil. All the major fatty acids identified by GC/MS as their methyl esters (Table 2) are represented in the TAG combinations identified by MS/MS (Table 3). Discrepancies between the two methods of analysis are found for eicosatrienoic (20:3n-3) and tetracosanoic (24:0) acids, which are not detected in the MS/MS analysis. It is likely that these two fatty acids were present in the minor TAG of clusters ACN 56–60, which were not analyzed by MS/MS. Hexadecadienoic acid (16:2), which comprised less than 0.01% of the total FA, was found by MS/MS in the MW fraction 852.7 Da. Each of the 14 MW species of TAG fraction analyzed were composed of 3–10 FA of various MW and number of double bonds. The MS/MS method was not able to distinguish between the positional isomers of double bonds of unsaturated fatty acids. The GC/MS results from the rapeseed FA methyl esters indicate that the only two FA in which this would cause a practical problem were 18:1n-9 and 18:1n-7.

The MS/MS spectra showed that most MW fractions consisted of mixtures of TAG. This restricted the ability to evaluate the regiospecific positions of fatty acids in the triacylglycerols. Nevertheless, it was possible to determine that in many TAG certain FA were preferentially (>67%) located at the *sn*-2 position, and these are labelled with superscript e in Table 3.

The major ion in the cluster ACN 50 (Fig. 4) corresponded to TAG of molecular weight 830.7 Da and was a mixture of 16:0/16:0/18:2 (62%), 16:1/16:0/18:1 (24%) and 14:0/18:1/18:1 (14%). Linoleic acid occupied more than 75% of the *sn*-2 position of the major TAG 16:0/16:0/18:2. The second biggest $[M - H]^-$ cluster in the NICI spectrum was ACN 52, which accounted for 18% of the total ion current. Four MW fractions from this cluster were analyzed further by MS/MS. These MW fractions contained TAG with two to five double bonds and from four to six different fatty acids. Two abundant TAG, 16:0-18:3-18:2 and 16:0-18:2-18:1, had linoleic acid preferentially at position *sn*-2. The major TAG of MW 858.7 Da was 16:0-18:1-18:1 (91%) and had 18:1 preferentially at the *sn*-2 position. The most abundant TAG cluster was ACN 54 (Table 3 and Fig. 4), which comprised close to 80% of turnip rapeseed oil TAG. TAG 18:3/18:2/18:1 and 18:2/18:2/18:1 represented over 20% of all the TAG in the seed oil, and the regioisomers 18:3-18:2-18:1 and 18:2-18:2-18:1 containing linoleic acid at *sn*-2 position, clearly dominated. The TAG of molecular weight 886.7 Da (ACN 54, 2 DB) consisted predominantly of FA combinations 18:1-18:1-18:0 (94%) and 18:2-18:0-18:0 (6%). There appeared to be a preference for 18:0 at the *sn*-2 position in the combination 18:1-18:1-18:0, *i.e.*, TAG *sn*-18:1-18:0-18:1 dominated. This is an interesting detail from a nutritional point of view, as the saturated *sn*-2-monoacylglycerols derived from these TAG have special melting and solubility properties (43,44). The MW species of 910.8 Da (ACN 56, 4 DB) contained six fatty acids. The major TAG of this MW was 18:2-18:1-20:1, which mainly occurred as the regioisomer 18:1-18:2-20:1. The ACN cluster 58 was extremely small due to the low erucic acid (22:1n-9) content of the "Kova" seeds. The major TAG fraction of ACN 58 had MW 940.8 Da and three double bonds. The complexity of this fraction prevented any regiospecificity from being determined.

Altogether, 44 different FA combinations of TAG were identified in the "Kova" variety, of which more than half existed in trace amounts (<1%). Results of the LEAR variety "Janpol" (40) based on GC analysis of FA, silver ion thin-layer chromatographic analysis of TAG and pancreatic lipase hydrolysis of the FA at positions *sn*-1 and *sn*-3 showed that linoleic acid preferentially occupies position *sn*-2 in several major TAG species. The same trend was reported earlier by Sergiel (45), although his results lacked information on specific triacylglycerols. The current status of LEAR varieties is summarized in detail by Ackman (29). The results obtained by NICI MS/MS are consistent with those of Zadernowski and Sosulski (40) with a few minor exceptions; namely, the major TAG source of stearic acid in "Kova" was 18:1/18:1/18:0 in which the saturated stearic acid preferentially occupied the *sn*-2 position. The MS/MS method also identified the TAG 18:2/18:2/18:2 to be present as 2–3% of the total TAG in "Kova oil."

CONCLUSIONS

The MS/MS method applied in this paper gives the opportunity to separate triacylglycerols with one dalton (*m/z*) resolution at the first quadrupole mass analyzer. The $[M - H]^-$ molecular ions may then be structurally characterized by a second mass analyzer. The advantages of the method developed include: (1) the sensitivity of the method (typically at subnanogram range of an individual TAG molecule); (ii) the method is extremely fast, *e.g.*, the time to characterize one MW fraction takes 10–15 min; (iii) the different MW species of TAG may be separated without any chromatographic steps; and (iv) the fatty acid combinations of each TAG and their regiospecific locations can be identified. The method does not replace the commonly accepted stereospecific analyses based on enzymatic hydrolysis, nor does it make the current chromatographic methods less valuable than they are today. However, the method may supplement the common methods of analysis and may be used as the first step in the characterization of TAG mixtures. At its present stage of development there are some limitations in its use, including: (i) the isomers of fatty acids (positions of double bonds, branching) cannot be distinguished; (ii) discrimination between the FA at positions *sn*-1 and *sn*-3 is not possible; and (iii) in complex TAG mixtures extraction of the *sn*-2 information is difficult or perhaps even impossible.

In the future, some of these limitations, including the difficulty of the MS/MS method in determining the *sn*-2 fatty acid in complex MW fractions, may be overcome by interfacing chromatographic procedures to the MS/MS system, *e.g.*, argentation high-performance liquid chromatography (46,47) or SFC (22,48). Other areas in which the method may prove useful are in on-line process control in the edible oil, dairy and pharmaceutical industries (32,33). The method is also applicable in enzyme methodology, when studying the regiospecificity of enzymes or in research on human nutrition and clinical applications.

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Triacylglycerols of Human Milk: Rapid Analysis by Ammonia Negative Ion Tandem Mass Spectrometry

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Human milk triacylglycerols (TAG) were analyzed by ammonia negative ion chemical ionization tandem mass spectrometry. The deprotonated molecular ions of triacylglycerols were fractionated at the first mass spectrometry (MS) stage. Twenty-nine of the deprotonated TAG ions were further analyzed based on their collisionally activated (CA) spectra. The tandem MS analysis covered eleven major acyl carbon number fractions, two of which contained odd carbon number fatty acids. Fatty acids of 28 different molecular weights were recorded from the daughter spectra. Hexadecanoic acid was present in all CA spectra, octadecenoic acid in the CA spectra of all mono- and higher unsaturated TAG, and octadecadienoic acid in the CA spectra of all di- and higher unsaturated TAG. The major fatty acid combinations in triacylglycerols were: with 0 double bonds (DB), 12:0/12:0/16:0; with 1 DB, 12:0/16:0/18:1; with 2 DB, 16:0/18:1/18:1; with 3 DB, 16:0/18:2/18:1; with 4 DB, 18:2/18:1/18:1; and with 5 DB, 18:2/18:2/18:1; hexadecanoic acid typically occupied the *sn*-2 position. The most abundant TAG was shown to be *sn*-18:1-16:0-18:1, comprising about 10% of all triacylglycerols.
Lipids 28, 217-222 (1993).

Triacylglycerols (TAG) comprise 98% of the total lipids in both human and cow milk (1). A thorough investigation of the molecular structures in the highly complex pool of triacylglycerols of human milk requires stepwise application of analytical procedures: (i) for the separation according to molecular weights and acyl carbon numbers; (ii) for the fractionation of each molecular weight cluster according to different fatty acid combinations; and (iii) for the analysis of the fatty acids in individual stereospecific positions. Mass spectrometry (MS) to complement chromatographic and enzymatic methods has been applied successfully (2-5). Freeman *et al.* (2) and later Kuksis and colleagues (6-8), as well as Weber *et al.* (9), have used chromatographic and mass spectrometric techniques to determine in detail the compositional and structural characteristics of human milk triacylglycerols.

Studies on human milk lipids have been exhaustively reviewed by Lammi-Keefe and Jensen (10), Davies *et al.* (11) and Jensen (12). In 1965, Freeman *et al.* (2) showed that in the triacylglycerols of human milk, *n*-hexadecanoic acid is typically located at the *sn*-2 position. According to Breckenridge *et al.* (7) the major acyl carbon number (ACN) species of human milk are, in descending order of abundance, ACN 52, 50, 54, 48, 46, 44, 56 and 42, although abundances may vary slightly depending on the days *postpartum* (9,13-15). Close to 100 TAG species have been identified in human milk so far (9).

The aim of this work was to apply a rapid tandem mass spectrometric method using ammonia negative ion chemical ionization (16,17) to human milk triacylglycerols in order to identify the most abundant fatty acid combinations in TAG. Information concerning the fatty acyl groups located preferentially at the *sn*-2 position also was obtained using the tandem mass spectrometry (MS/MS) method.

MATERIALS AND METHODS

Human milk triacylglycerols. A sample of milk was obtained from a young Australian mother 1 day *postpartum*. Lipids were extracted according to the method of Bligh and Dyer (18) using $CHCl_3$/MeOH.

Tandem mass spectrometric analysis. The lipid fraction of human milk was dissolved in dichloromethane (*ca.* 1 mg/mL), and 1 μL of this mixture was placed in an aluminum crucible located at the end of the standard direct insertion probe of a Finnigan MAT (San Jose, CA) TSQ-70 triple quadrupole mass spectrometer. The negative ion chemical ionization mass spectra of TAG were acquired using ammonia as the reagent gas, and the collisionally activated (CA) daughter spectra of the deprotonated TAG were obtained using argon as the collision gas (17). The method, its mass spectral basis and its applicability have been described in more detail previously (17,19).

The CA daughter spectra of the $[M - H]^-$ ions consisted of two main fragment ion clusters, namely RCO_2^- and $[M - H - RCO_2H - 100]^-$ (17,19). These fragment ions were used to deduce the structure of the $[M - H]^-$ ion and thus of the TAG molecule(s). The proportions of fatty acids within the deprotonated TAG of each parent ion were calculated according to the intensities of the RCO_2^- ions corrected by empirical molar correction factors. The loss of the $(RCO_2H + 100)$ fragments from the $[M - H]^-$ ion in a CA daughter spectrum was used to obtain information regarding the fatty acyl groups at the *sn*-2 position, as shown previously (17,19).

RESULTS AND DISCUSSION

The triacylglycerols of human milk were deprotonated in the gas phase using ammonia negative ion chemical ionization (NICI). The individual deprotonated molecular weight fractions, $[M - H]^-$, were selected one by one using the first quadrupole mass spectrometer of the TSQ-70 instrument. This method allowed the separation of TAG species of human milk by mass with a practical resolution of 1 amu. The aim and goal of this NICI MS stage was analogous to currently available chromatographic techniques commonly used prior to single stage MS analysis. Mass spectral separation of the $[M - H]^-$ ions is, however, more efficient than is currently practical by chromatographic techniques. The $[M - H]^-$ ions chosen were then collisionally activated in the second quadrupole with argon, and the mass spectrum of the fraction was

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Abbreviations: ACN, acyl carbon number; CA, collisional activation; DB, double bonds; FA, fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; NICI MS, negative ion chemical ionization mass spectrometry; TAG, triacylglycerols.

obtained by scanning with the third quadrupole. No selectivity according to chemical or physical properties other than molecular weight was noted by MS.

Any signal corresponding to an $[M - H]^-$ ion would also contain some ions which originate from TAG having two ^{13}C atoms randomly distributed in their carbon skeleton but containing one additional double bond in their fatty acyl residues. The proportions of these isotopic contributions were subtracted from the $[M - H]^-$ parent ion. The corrected values for the even ACN TAG are summarized in Figure 1.

The major $[M - H]^-$ cluster in the NICI mass spectrum (Fig. 1) was ACN 52 (isotope-corrected proportion, 23%) followed by ACN 46–50 (*ca.* 16% each), ACN 44 (11%), ACN 42 (7%), ACN 40 (5%), ACN 54 (5%) and ACN 38 (3%). The corrected proportions of the saturated, monoenoic, dienoic, trienoic, tetraenoic and pentaenoic TAG were 14, 38, 30, 14, 4 and 1%, respectively (Fig. 1).

The specific responses of $[M - H]^-$ ions which varied according to their molecular weights were not taken into account in Figure 1 due to lack of proper correction factors. It was recognized that the lowest molecular weight areas were always overestimated in ammonia NICI MS when compared with the high mass range. The differing volatilities of triacylglycerols, which differ according to molecular weight, were therefore taken into account by acquiring the NICI spectra over a wide range and by averaging the spectra (17). Both the proportions of ACN species and of TAG with different degrees of unsaturation were close to earlier reports (12,15). Due to the effect of the mother's diet, especially in regard to the linoleic acid content of the milk, average values can only be indicative (20).

Examination of Figure 1 shows that the degree of unsaturation in TAG increases as the molecular weight of the TAG increases. This is in agreement with the earlier observations by Breckenridge *et al.* (7), who used a combination of chromatographic techniques in their studies. The majority of the TAG were mono- and diunsaturated, and the saturated TAG were not observed in notable quantities in ACN larger than 48. All ACN species with 48 or more acyl carbons contained tetraenoic TAG species. Pentaenoic TAG were found in all ACN groups with at least 52 acyl carbons. Those MW species with at least 0.5% relative abundance of total TAG (Fig. 1) were further analyzed by CA MS/MS. Although small traces of ACN species beyond 38–54 were observed, they were not analyzed. Several minor TAG with odd ACN could be observed between the major even-numbered TAG by NICI MS. However, only two of these odd-numbered species, 762.7 and 790.7, were further analyzed by tandem MS.

Table 1 lists the 28 fatty acid species identified based on both the RCO_2^- and $[M - H - RCO_2H - 100]^-$ ions in the CA daughter spectra of the $[M - H]^-$ ions. It is important to note that in these experiments no information other than the molecular weights of the fatty acids could be obtained, *i.e.*, information such as the positions and configurations of double bonds or possible branching could not be deduced. Thus, the minor TAG species containing *trans*-acids, such as 14:1, 16:1, 17:1 or 18:1, cannot be distinguished from the corresponding major *cis*-acids, nor can 18:3n-6 (γ-linolenic acid) and 18:3n-3 (α-linolenic acid) be separated without silver ion high-performance liquid chromatography (HPLC) (21) or an equivalent fractionation method. The fatty acid composition

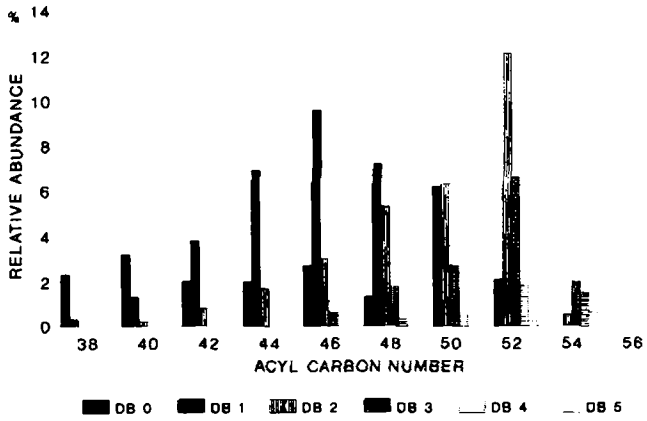

FIG. 1. Distribution of triacylglycerols in human milk according to the acyl carbon numbers and number of double bonds (DB). Calculations are based on the intensities of the ^{13}C isotope corrected $[M - H]^-$ ions of ammonia negative ion chemical ionization mass spectra.

of human milk is, however, well known (*e.g.*, 6,7,12,22, 23).

The daughter spectra of the $[M - H]^-$ ions revealed that hexadecanoic acid (16:0) was present in each molecular weight species of the TAG analyzed, octadecenoic acid (18:1) in each unsaturated species, and octadecadienoic acid (18:2) in each di- and higher unsaturated ones. Because of the lack of some response correction factors, the relative abundances of the fatty acids are not listed in Table 1.

Dodecanoic (12:0) and tetradecanoic (14:0) acids are typical constituents of human milk, and they were present in all TAG species up to MW 832.7 (ACN 50, 1 DB) and MW 860.7 (ACN 52, 1 DB), respectively. Octanoic acid (8:0) is not listed in Table 1 because the ion corresponding to the deprotonated fatty acid was not observed in the CA spectra, although the presence of this acid was indicated by the loss of $(RCO_2H + 100)$ of the $[M - H]^-$ ions. The absence of RCO_2^- ions derived from octanoic acid may be due to the larger potential energy loss in the fragmentation process; this could result in poor transmission through the third quadrupole and, hence, in a weak ion signal or no signal at all. Hexanoic (6:0) and butanoic (4:0) acids, which are known to be present in extremely small quantities in TAG evidently smaller than ACN 38, were not observed in the parent ions analyzed.

Long-chain fatty acids, such as 20:5, 22:4, 22:5, 22:6, 24:0 and 24:1, are typically present in TAG of ACN 56 and higher (6,22). However, the fatty acids were not found in the MW species analyzed by collisional activation. Yet, traces of the clusters ACN 56 and ACN 58 could be seen in the NICI mass spectrum of human milk TAG. The 29 MW species analyzed by MS/MS indicated that these MW fractions comprised 277 different TAG, even when the positional isomers and different fatty acid isomers are excluded. The two most abundant TAG of each MW fraction are listed in Table 2. Information concerning the number of various fatty acids and TAG within each MW species is also given. The fatty acids marked with a ^{c} were estimated, based on the relative abundances of the $[M - H - RCO_2H - 100]^-$ ions in the CA daughter spectra, to be preferentially located at position *sn*-2 (17). The

TABLE 1

Distribution of Fatty Acids in Human Milk Triacylglycerols Analyzed by Collisional Activation of the $[M-H]^-$ Ions

			Fatty acids[d]												
ACN[a]	MW[b]	DB[c]	10:0	12:0	14:0	16:0	18:0	20:0	11:0	13:0	15:0	17:0	10:1	12:1	14:1	16:1
38	666.7	0	x	x	x	x	x									
40	692.7	1	x	x	x	x	x						x	x	x	x
40	694.7	0	x	x	x	x	x									
42	718.7	2	x	x	x	x	x						x	x	x	x
42	720.7	1	x	x	x	x	x								x	x
42	722.7	0	x	x	x	x	x									
44	746.7	2	x	x	x	x	x						x	x	x	x
44	748.7	1	x	x	x	x	x								x	x
44	750.7	0	x	x	x	x	x									
45	762.7	1	x	x	x	x	x		x	x	x	x			x	x
46	774.7	2	x	x	x	x	x							x	x	x
46	776.7	1	x	x	x	x	x								x	x
46	778.7	0		x	x	x	x									
47	790.7	1	x	x	x	x	x			x	x	x			x	x
48	800.7	3		x	x	x	x							x	x	x
48	802.7	2		x	x	x	x								x	x
48	804.7	1		x	x	x	x	x							x	x
48	806.7	0		x	x	x	x	x								
50	828.7	3		x	x	x	x								x	x
50	830.7	2		x	x	x	x								x	x
50	832.7	1		x	x	x	x	x								x
52	854.7	4			x	x	x									x
52	856.7	3			x	x	x									x
52	858.7	2			x	x	x									x
52	860.7	1			x	x	x	x								
54	880.7	5				x	x									
54	882.7	4				x										
54	884.7	3				x	x									
54	886.7	2				x	x	x								x

			18:1	20:1	22:1	13:1	15:1	17:1	19:1	16:2	18:2	20:2	22:2	18:3	20:3	20:4
38	666.7	0														
40	692.7	1	x													
40	694.7	0														
42	718.7	2	x	x						x	x					
42	720.7	1	x													
42	722.7	0														
44	746.7	2	x	x						x	x	x				
44	748.7	1	x	x												
44	750.7	0														
45	762.7	1	x			x	x	x	x							
46	774.7	2	x	x						x	x	x	x			
46	776.7	1	x	x												
46	778.7	0														
47	790.7	1	x	x			x	x	x							
48	800.7	3	x							x	x	x			x	
48	802.7	2	x								x	x				
48	804.7	1	x	x	x											
48	806.7	0														
50	828.7	3	x	x						x	x	x		x	x	
50	830.7	2	x	x							x	x				
50	832.7	1	x	x												
52	854.7	4	x								x	x		x	x	x
52	856.7	3	x								x	x		x	x	
52	858.7	2	x	x							x	x				
52	860.7	1	x													
54	880.7	5	x								x			x	x	x
54	882.7	4	x	x							x			x		
54	884.7	3	x	x							x	x			x	
54	886.7	2	x	x							x	x				

[a]Even acyl carbon number (ACN) species exceeding 0.5% relative abundances in Figure 1, and two of the major odd acyl carbon number species (ACN 45 and ACN 47) are included only.
[b]Molecular weight of triacylglycerol.
[c]Number of double bonds in triacylglycerol.
[d]Molecular weights of the fatty acids are known only. Identifications are based on both RCO_2^- and $[M-H-RCO_2H-100]^-$ ions of collisionally activated daughter spectra of deprotonated molecular ions.

TABLE 2

Major Triacylglycerols (TAG) in each of the Molecular Weight Species Analyzed by Collisional Activation of the Deprotonated Molecular Ions

ACN[a]	MW[b]	DB[c]	Major TAG	Second most abundant TAG	Number of FA[d]	Number of TAG[e]
38	666.7	0	12:0/12:0/14:0	10:0/12:0/16:0	5	4
40	692.7	1	10:0/12:0/18:1	10:0/14:0/16:1	10	9
40	694.7	0	12:0/12:0/16:0[f]	12:0/14:0/14:0	5	4
42	718.7	2	12:0/12:0/18:2	10:0/14:0/18:2	13	17
42	720.7	1	12:0/12:0/18:1	10:0/14:0[f]/18:1	8	7
42	722.7	0	12:0/14:0/16:0[f]	12:0/12:0/18:0	5	5
44	746.7	2	12:0/14:0[f]/18:2	10:0/16:0[f]/18:2	14	18
44	748.7	1	12:0/14:0[f]/18:1	10:0/16:0[f]/18:1	9	9
44	750.7	0	12:0/16:0/16:0	12:0/14:0/18:0	5	4
45	762.7	1	12:0/15:0[f]/18:1	12:0/16:0[f]/17:1	16	28
46	774.7	2	12:0/16:0[f]/18:2	12:0/16:1/18:1	14	20
46	776.7	1	12:0/16:0[f]/18:1	14:0/14:0/18:1	9	9
46	778.7	0	12:0/16:0[f]/18:0	14:0/16:0[f]/16:0	4	2
47	790.7	1	14:0/15:0/18:1	12:0/17:0/18:1	14	23
48	800.7	3	12:0[f]/18:2/18:1	14:0/16:0[f]/18:3	13	14
48	802.7	2	12:0/18:1/18:1	14:0/16:0/18:2	10	11
48	804.7	1	14:0/16:0[f]/18:1	12:0/18:1/18:0	10	10
48	806.7	0	14:0/16:0[f]/18:0	16:0[f]/16:0/16:0	5	5
50	828.7	3	14:0[f]/18:2/18:1	16:1/16:0[f]/18:2	13	15
50	830.7	2	14:0[f]/18:1/18:1	16:0[f]/16:0/18:2	10	12
50	832.7	1	16:0[f]/16:0/18:1	14:0[f]/18:1/18:0	9	7
52	854.7	4	16:0[f]/18:2/18:2	16:0[f]/18:3/18:1	10	9
52	856.7	3	16:0[f]/18:2/18:1	16:1/18:2/18:0	9	8
52	858.7	2	16:0[f]/18:1/18:1	16:0[f]/18:2/18:0	8	7
52	860.7	1	16:0[f]/18:1/18:0	14:0/18:1/20:0	5	2
54	880.7	5	18:2/18:2/18:1	16:0[f]/18:1/20:4	7	5
54	882.7	4	18:2/18:1/18:1	16:0/18:3/20:1	5	2
54	884.7	3	18:1/18:1/18:1	18:2/18:1/18:0	8	7
54	886.7	2	18:1/18:1/18:0	16:0/18:1/20:1	7	4

[a]Acyl carbon number.
[b]Molecular weight.
[c]Number of double bonds in triacylglycerols.
[d]Identifications are based on both RCO_2^- and $[M-H-RCO_2H-100]^-$ ions of collisionally activated daughter spectra of the deprotonated triacylglycerols. FA, fatty acid.
[e]The number of FA combinations in TAG.
[f]Preferentially at *sn*-2 position.

MS/MS results are consistent with the currently accepted concept that in human milk TAG hexadecanoic acid is typically located at position *sn*-2 (2,7,24,25). It could be seen from our data that in several instances where 16:0 acid was not present, 14:0, 15:0 and even 12:0 preferentially took the place of 16:0 and occupied the *sn*-2 position (Table 2). Although Freeman *et al.* (2) and Breckenridge *et al.* (7) observed the preference of 16:0 for *sn*-2 more than twenty years ago, they were not able to do this at the level of individual TAG species. The advantage of the MS/MS method is that direct information can be obtained on the regiospecific structure of individual TAG species (or small groups) instead of having to rely on averaged results of enzymatic analyses carried out on complex mixtures. In addition, the MS/MS method is more rapid.

A recent analysis of human milk by HPLC and gas chromatography (GC) reported by Weber *et al.* (9) identified and quantified 94 triacylglycerols in human milk, although no information regarding the stereospecific distribution of the fatty acids was given. All the TAG listed in Table 2, except for the two odd-numbered fatty acid TAG species and 12:0/12:0/18:2, also were reported by Weber *et al.* (9). For example, the typical CA daughter spectra of the two major MW species 858.7 (ACN 52, 2 DB) and MW 776.7 (ACN 46, 1 DB) are shown in Figures 2a and 2b, respectively. Examination of Figure 2a shows that the $[M-H]^-$ ion with the recorded *m/z* 857.4 in the NICI spectrum consisted of eight fatty acids (eight different MW species of fatty acids). The number of fatty acid combinations of the TAG was seven, based on the known number of carbons (ACN 52) and the degree of unsaturation (2 DB) in each TAG species (17).

According to the proportions of the RCO_2^- ions in Figure 2a, the order of abundances of the TAG in this MW fraction are 16:0/18:1/18:1, 16:0/18:2/18:0, 16:1/18:1/18:0, 16:1/16:0/20:1, 14:0/18:1/20:1, 16:0/16:0/20:2 and 14:0/18:0/20:2. The order of the fatty acids (separated by slashes) does not imply that the stereospecific positions of the fatty acyl groups are known. The two major TAG combinations of the MW fraction *m/z* 857.4 comprised more than 95% of the seven TAG species present, based on the relative counts of the RCO_2^- ions. From the abundances of the $[M-H-RCO_2H-100]^-$ fragment ions, it is clear (17,19) that hexadecanoic and tetradecanoic acids were preferentially located at the *sn*-2 position. Within the $[M-H-RCO_2H-100]^-$ cluster, the relative abundance of the signal originating from the 16:0

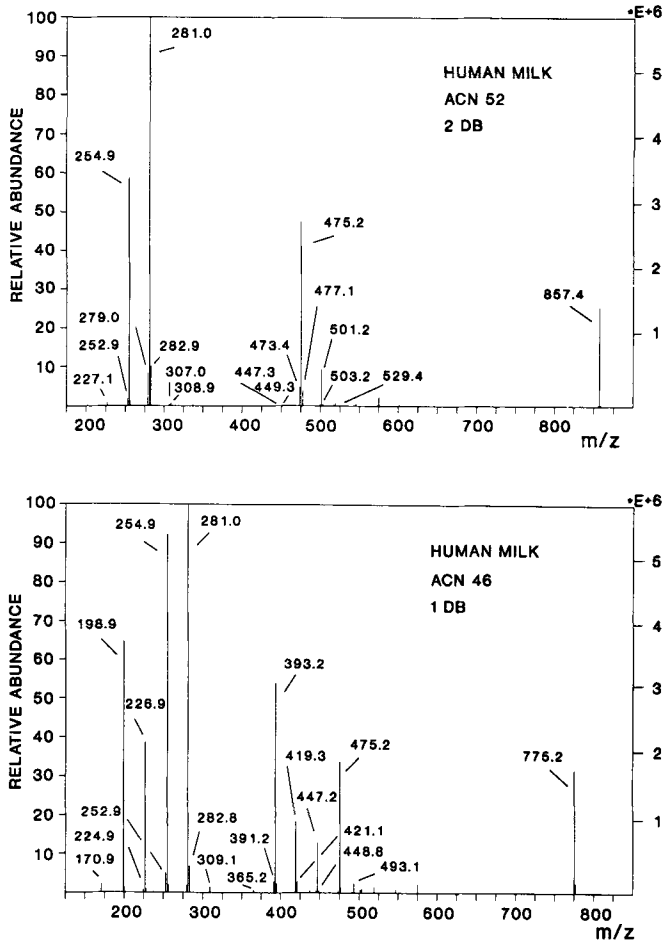

FIG. 2. Collisionally activated daughter spectra of the deprotonated molecular ions of triacylglycerols of molecular weights 858.7 (a) and 776.7 (b) from human milk.

fatty acid (recorded *m/z* 501.2) was only 14.2%, whereas within the RCO_2^- cluster the corresponding abundance of 16:0 (recorded *m/z* 254.9) was 32.3%.

To accurately calculate the proportions of fatty acids and their primary *vs.* secondary positions in the TAG species (*e.g.*, Fig. 2a), precise calibration for all the RCO_2^- ions is required. If just the three major TAG species—16:0/18:1/18:1, 16:0/18:2/18:0 and 16:1/18:1/18:0—were taken into account and the earlier defined correction factors (17) were applied ($k_{18:0} = 1.0$, $k_{18:1} = 1.3$, $k_{18:2} = 1.6$, $k_{16:0} = 1.1$ and $k_{16:1} = 1.5$), the relative proportions of the three TAG were 86, 12 and 2%, respectively. It could then be determined that 85% of 16:0 acid in the major TAG of human milk, 16:0/18:1/18:1, was located at position *sn*-2 (17). Thus the most abundant TAG in human milk, comprising 9% of all TAG species, was shown to be *sn*-18:1/16:0/18:1. The absolute error may not be very high, as the fatty acids excluded from the calculations above, 14:0, 20:1 and 20:2, comprised only 1-2% of the total fatty acid pool of the molecular weight 858.7 TAG.

The CA daughter spectrum of the MW fraction 776.7 (Fig. 2b, measured *m/z* $[M - H]^- = 775.2$) showed that this fraction consisted of nine different fatty acids, which again originated from nine different TAG species. The two most abundant TAG based on a relative ion count of the RCO_2^- ions were 12:0/16:0/18:1 and 14:0/14:0/18:1. The other TAG were 10:0/16:0/20:1, 10:0/18:1/18:0, 12:0/14:0/20:1, 12:0/16:1/18:0, 14:1/14:0/18:0, 14:0/16:1/16:0 and 14:1/16:0/16:0.

The list of the most abundant TAG species in each MW fraction (Table 2) and the distribution of TAG in Figure 1 are quite consistent with the results of Watts and Dils (14), as well as those of the Kuksis group (6-8). Their results, however, showed a slightly different order of relative abundances of the major TAG when compared with our data; *e.g.*, the total proportion of ACN 52 TAG, 23%, was lower than that reported by other authors. This discrepancy may suggest an underestimation of the abundances of the higher MW TAG by the ammonia NICI method. It is, however, quite possible that a number of natural factors may account for this difference, *e.g.*, randomization in human milk, which is known to occur as lactation proceeds (14), and the susceptibility of human milk TAG to dietary habits (7,26).

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Hypolipidemic Activity of 6-Alkoxycarbonyl-3-aryl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones and 2-Alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1H,5H)-diones in Rodents

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Significant hypolipidemic activity was demonstrated by 6-ethoxycarbonyl-3-phenyl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-dione, 2-ethoxycarbonyl-5-phenyl-1,3,5-triazine-4,6(1H,5H)-dione and 2-ethoxycarbonyl-5-(4-chlorophenyl)-1,3,5-trizine-4,6(1H,5H)-dione in rodents at 20 mg/kg/day. These agents lowered serum cholesterol and triglyceride levels by approximately 40% in mice after 16 d. Tissue lipids in rat liver, small intestinal mucosa, aortic wall and feces were reduced by treatment with the agents. Very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol levels were reduced in the rat; high density lipoprotein (HDL) cholesterol levels were elevated after 14 d of treatment. The activities of regulatory enzymes, *e.g.*, acetyl-CoA synthetase, acyl-CoA:cholesterol acyltransferase, cholesterol 7α-hydroxylase, *sn*-glycerol-3-phosphate acyltransferase, phosphatidylate phosphohydrolase and heparin-induced lipoprotein lipase, involved in *de novo* synthesis of hepatic lipids were affected by the agents. The new compounds may represent another class of potentially useful hypolipidemic agents for the treatment of atherosclerosis since HDL cholesterol levels were increased and VLDL and LDL cholesterol levels were lowered by some of the agents.

Lipids 28, 223–229 (1993).

We have previously reported that a number of low molecular weight compounds having different ring structures, *e.g.*, succinimides (1), dilantin (2), phenobarbital (3), terephthalic acid (4), 2-pyrrolidinones (5,6), 4-pyrimidinecarboxylic acids (7), 2-furoic acid (8) and 3,4-diphenylpiperidine-2,6-diones (9), have potent hypolipidemic activity in rodents. These agents were effective in lowering both serum cholesterol and triglyceride levels by 30–40% in rodents. Substitutions on the nitrogen atom of the ring, *e.g.*, cyclic imides with a bulky group [*N*-(4-methylphenyl)-diphenimide and *o*-(*N*-phthalimido)acetophenone], led to compounds which significantly lowered very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol and elevated high density lipoprotein (HDL) cholesterol levels in rats at 10–20 mg/kg/d when administered orally. Subsequently, series of 1-acylated and 1,2-diacylated 4-substituted 1,2,4-triazolidine-3,5-dione and 2-benzoyl-4,4-dialkyl-3,5-isoxazolidinediones have demonstrated potent hypolipidemic activities in rodents (10,11). Since the earlier compounds have some structural similarities with 6-alkoxycarbonyl-3-aryl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones and 2-alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1H,5H)-diones, we tested the new derivatives for hypolipidemic activity in rodents. Results of these studies are reported here.

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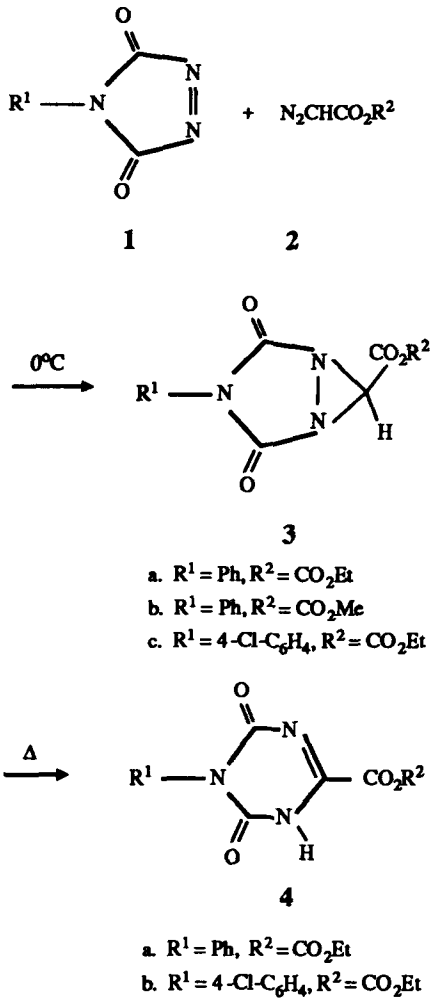
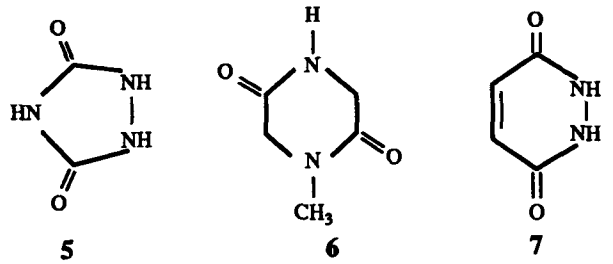
Abbreviations: EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; SAR, structure-activity relationship; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

The synthesis and the physical, chemical and spectral properties of 6-ethoxycarbonyl-3-phenyl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-dione (**3a**), 6-methoxycarbonyl-3-phenyl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-dione (**3b**), 6-ethoxycarbonyl-3-(4-chlorophenyl)-1,3,5-triazabicyclo-[3.1.0]hexane-2,4-dione (**3c**), 2-ethoxycarbonyl-5-phenyl-1,3,5-triazine-4,6(1H,5H)-dione (**4a**) and 2-ethoxycarbonyl-5-(4-chlorophenyl)-1,3,5-triazine-4,6(1H,5H)-dione (**4b**) have been described previously (Scheme 1) (10,11). Compounds **5**, **6** and **7** were purchased from Aldrich Chemical Co. (Milwaukee, WI) (Scheme 2). All radioactive compounds were purchased from New England Nuclear (DuPont, Boston, MA). Substrates and cofactors for the enzyme assays were obtained from Sigma Chemical Co. (St. Louis, MO).



SCHEME 1



5 6 7

SCHEME 2

Hypolipidemic screens in normal rodents. Test compounds **3a–c, 4a–b, 5, 6** and **7** were suspended in an aqueous 1% carboxymethylcellulose solution, homogenized and administered to CF_1 male mice (~25 g) at 20 mg/kg/d intraperitoneally for 16 d. Structure-activity relationship (SAR) studies were performed at 20 mg/kg/d for 16 d because this dose was optimal in activity for the succinimide, 2-furoic acid, dilantin, glutamide and phenobarbital studies in rodents (1–9). On days 9 and 16, blood was obtained from the tail vein, and serum was separated by centrifugation at 3500 × *g* for 3 min. Serum cholesterol levels were determined by a modification of the Liebermann-Burchard reaction (12). Serum was also analyzed for triglyceride content as determined by a commercial kit (BioDynamics/bmc single vial, triglycerides colorimetric method 348201, Boehringer Mannheim Diagnostics, Indianapolis, IN). Food and water were available *ad libitum.* The animals were maintained at 22°C on Rat, Mouse, Hamster 3000, Agway ProLab Animal Diets (Syracuse, NY) under a 12-h light/dark cycle.

Hyperlipidemic induced CF_1 mice. CF_1 male mice (~30 g) were fed a 1:1 mixture of basal atherogenic diet (U.S. Biochemical Corp., Cleveland, OH) with ground rodent chow (ProLab Animal Diet) for 14 d. In the diet-induced hyperlipidemic mice, serum cholesterol was elevated from normal levels of 121 mg% to 361 mg% whereas the serum triglyceride levels were elevated from normal levels of 137 mg% to 357 mg% over a two-week period. Drug administration was commenced at 20 mg/kg/d *i.p.* and continued for the next 14 d. Hyperlipidemic mice were bled and serum cholesterol and triglycerides were measured as previously described (3).

Sprague-Dawley rats. Selected compounds **3a, 4a, 4b** and **7** were tested in Sprague-Dawley male rats (~280 g) by oral administration at 20 mg/kg/d for 14 d maintained on ProLab Animal diet *ad libitum.* Serum cholesterol and triglyceride levels were measured as previously described.

Animal weights and food intake. Food consumption was determined daily as gram food/rat/d for control rats which were maintained on ProLab Animal diet and rats treated orally with compounds **3a, 4a, 4b** and **7** at 2 mg/kg/d. Body weights were obtained on days 7 and 14; they were expressed as percentage of the animal's weight on day 0. After dosing for 14 d with compounds, selected organs were excised, trimmed of fat and weighed (3).

Liver, small intestine, aorta and fecal lipid extraction. In Sprague-Dawley male rats on ProLab Animal diet receiving the test compounds **3a, 4a, 4b** and **7** at 20 mg/kg/d, orally for 14 d, the liver, small intestinal mucosa from duodenum to ileum, aorta and fecal materials (24-h collection) were removed and weighed. Homogenates (10%) (wt/vol) were prepared, extracted (13,14) and analyzed for cholesterol (12), triglyceride, neutral lipid (15) and phospholipid (16) contents. Protein content of the whole homogenate was also determined (17).

Enzymatic studies. Enzymatic assays were carried out using 10% homogenates of CF_1 male mouse liver prepared in 0.25M sucrose and 0.001M ethylenediaminetetraacetic acid (EDTA) (pH 7.2) with 25–100 μM concentrations of compounds **3a, 4a, 4b** and **7** *in vitro.* Activities of the following enzymes were determined by using established procedures: acetyl-coenzyme A synthetase (18); adenosine triphosphate dependent citrate lyase (19); mitochondrial citrate exchange (20,21); cholesterol 7α-hydroxylase (22); 3-hydroxy-3-methylglutaryl-coenzyme A reductase (23,24); acetyl-coenzyme A carboxylase (25); neutral cholesterol ester hydrolase (26); *sn*-glycerol-3-phosphate acyltransferase (27); phosphatidylate phosphohydrolase activity (28); acyl-CoA cholesterol acyltransferase (29) and heparin-activated hepatic lipoprotein lipase (30). Protein in the whole homogenates and in the mitochondrial and microsomal fractions was assayed according to Lowry *et al.* (17).

Serum lipoprotein fractionation. Sprague-Dawley male rats maintained on ProLab Animal diet (~340 g) were administered compounds **3a, 4a** and **4b** or **7** at 20 mg/kg/d orally. Blood was collected from the abdominal vein, and lipoprotein fractions were obtained by the methods of Hatch and Lees (31), and Havel *et al.* (32) as modified for the rat (33). Each fraction was analyzed for cholesterol (12), triglyceride, neutral lipids (15), phospholipids (16) and protein (17). LDL and HDL lipoprotein fractions were dialyzed, delipidated, and the apoproteins were separated by SDS-PAGE (5–15%) electrophoresis on 3% acrylamide gel which was stained with 0.5% coomassie blue (34).

RESULTS

We have recently described the synthesis of 6-alkoxycarbonyl-3-aryl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones (**3**) and 2-alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1H,5H)-diones (**4**) by the reaction sequence shown in Scheme 1 (10,11). This method was used to prepare compounds **3a–c** and **4a–b**. Compounds **3** were obtained by reaction of 4-substituted 1,2,4-triazole-3,5(4H)-diones (**1**) with alkyl diazoacetates (**2**) in dichloromethane at 0°C. These compounds are white amorphous solids composed of monomers which associate in solution. The extent of association increases as the temperature is lowered and as the (**3**) ⇌ $(3)_n$ ($n \geq 2$) concentration of the solutions is increased. Heating compounds **3** in chlorobenzene under reflux for two weeks resulted in ring-opening to give compound **4**.

Pharmacology. Compounds **3a, 3c, 4a** and **4b** tested in CF_1 mice at 20 mg/kg/d showed similar hypocholesterolemic activity, *i.e.,* a 43–46% reduction (Table 1). Compounds **3a, 4a** and **4b** demonstrated a 41–69% reduction of serum triglyceride levels. Compound **3b** caused only a 28% reduction of serum cholesterol and a 31% reduction of triglyceride levels. Compound **3c** was not as active showing only a 34% reduction of serum triglyceride levels. Compounds **5, 6** and **7** were not as effective as hypolipidemic agents in mice. Compounds **3a, 4a** and **4b** in hyperlipidemic diet induced mice successfully reduced elevated serum cholesterol levels by 51–56% (Table 2).

TABLE 1

The Effects of 6-Alkoxycarbonyl-3-aryl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones and 2-Alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1H,5H)-diones on CF_1 Male Mouse Serum Lipid Levels After Sixteen Days Administration of Drugs (i.p.)

N = 6 Compounds	Control (%) Serum cholesterol Day 9	Serum cholesterol Day 16	Serum triglyceride Day 16
3a	62 ± 6	57 ± 5[a]	59 ± 4[a]
3b	76 ± 7	72 ± 5	69 ± 6
3c	74 ± 7	56 ± 4[a]	66 ± 5[a]
4a	64 ± 7	54 ± 4[a]	31 ± 4
4b	63 ± 5	57 ± 6[a]	55 ± 4[a]
5	81 ± 6	79 ± 4[a]	73 ± 6[a]
6	70 ± 5	63 ± 5[a]	75 ± 5[a]
7	74 ± 6	66 ± 6[a]	75 ± 4[a]
Control 1% carboxymethyl cellulose	100 ± 7[b]	100 ± 6[c]	100 ± 7[d]
Clofibrate	88 ± 7	86 ± 6	75 ± 5[a]

[a]$P < 0.001$. [b]125 mg%. [c]128 mg%. [d]137 mg%.

TABLE 2

The Effects of Compounds 3a, 3c, 4a, 4b and 7 [20 mg/kg/d (i.p.)] on Serum Lipids of Hyperlipidemic Induced CF_1 Mice

N = 6 Compounds	Control (%) Serum cholesterol	Serum triglyceride
3a	46 ± 4[a]	54 ± 5[a]
3c	62 ± 6[a]	59 ± 5[a]
4a	44 ± 4[a]	47 ± 4[a]
4b	49 ± 5[a]	46 ± 6
7	51 ± 6[a]	50 ± 7[a]
Control 1% carboxymethyl cellulose	100 ± 6[b]	100 ± 7[c]

[a]$P \leq 0.001$. [b]362 mg%. [c]357 mg%.

Elevated serum triglycerides levels in these mice were reduced by 54–56% by these three compounds.

In Sprague-Dawley rats, **3a** at 20 mg/kg/d showed good activity, lowering serum cholesterol and serum triglycerides by 64 and 46%, respectively, by days 7 and 14 (Table 3). Compound **4a** (10 mg/kg/d) reduced serum cholesterol levels by 40%, but reduced serum triglyceride levels by only 23% by day 14. Compound **4b** (20 mg/kg/d) reduced serum cholesterol levels by 33% and serum triglyceride levels by 47% by day 14. Compound **7** caused a 24% reduction of serum cholesterol and a 32% reduction of serum triglyceride on day 14. In rats, none of the compounds suppressed food intake on a daily basis. The body weights did not differ from the control weights over 14 d. Organ weights of treated rats were not affected by the 14-d drug treatment.

Examination of tissue lipids showed that **4b** and **7** lowered liver and aorta wall cholesterol and neutral lipid levels. Compound **3a** lowered small intestinal mucosa cholesterol, triglyceride and protein contents (Table 4). Aorta cholesterol was reduced moderately by 18% by **3a**. Compound **4a** at 10 mg/kg/d elevated cholesterol levels in the liver and small intestinal mucosa, but lowered aorta wall cholesterol levels. The level of phospholipids was lowered in the small intestinal mucosa by **4a** and **4b**. Fecal lipids were increased by **3a** and **4b**. Treatment with **3a** resulted in significant increases in fecal cholesterol, triglyceride, phospholipid and protein contents. Treatment with **4b** resulted only in an increase in fecal cholesterol levels. Compound **7** caused moderate increases in fecal cholesterol, neutral lipid and phospholipid levels.

The serum lipoprotein lipid content was also modulated by the drugs (Table 5). Compounds **3a** and **4b** reduced

TABLE 3

The Effects of Compounds 3a, 4a, 4b and 7 Orally Administered on the Serum Lipid Levels of Male Sprague-Dawley Rats

N = 6 Compounds	Control (%) Serum cholesterol Day 7	Serum cholesterol Day 14	Serum triglyceride Day 7	Serum triglyceride Day 14
3a 20 mg/kg/d	70 ± 5[a]	36 ± 4[a]	36 ± 5[a]	54 ± 6[a]
4a 10 mg/kg/d	84 ± 7	60 ± 4[a]	82 ± 4	77 ± 7[a]
4b 20 mg/kg/d	71 ± 6[a]	67 ± 6[a]	61 ± 5[a]	53 ± 5[a]
7 20 mg/kg/d	92 ± 5	76 ± 7[a]	90 ± 4	68 ± 6[a]
Control 1% carboxymethyl cellulose	100 ± 6[b]	100 ± 7[c]	100 ± 8[d]	100 ± 6[e]
Clofibrate 150 mg/kg/d	89 ± 7	86 ± 5	83 ± 6	74 ± 5
Lovastatin 8 mg/kg/d	85 ± 4	82 ± 5	91 ± 5	86 ± 7

[a]$P \leq 0.001$. [b]75 mg%. [c]78 mg%. [d]110 mg%. [e]112 mg%.

TABLE 4

The Effects of Hypolipidemic Agents on Tissue Lipids of Sprague-Dawley Rats Administered Orally at 10 or 20 mg/kg for 14 d[a]

	Control (%)					
N = 6 Compound	Mg of lipid extracted	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Protein
Liver						
3a	91 ± 7	105 ± 5	80 ± 7[b]	99 ± 7	87 ± 8	91 ± 6[b]
4a	119 ± 7	135 ± 8	109 ± 7	67 ± 5[b]	105 ± 6	98 ± 5
4b	90 ± 6	78 ± 3[b]	89 ± 6	79 ± 6[b]	121 ± 7	113 ± 6
7	79 ± 6[b]	82 ± 6[b]	95 ± 5	71 ± 6[b]	113 ± 8	100 ± 6
Control	100 ± 7[c]	100 ± 6[d]	100 ± 8[e]	100 ± 7[f]	100 ± 8[g]	100 ± 5[h]
Small intestinal mucosa						
3a	50 ± 5[b]	38 ± 5[b]	36 ± 5[b]	103 ± 6	112 ± 7	47 ± 4[b]
4a	176 ± 8	150 ± 7[b]	87 ± 5	61 ± 6[b]	36 ± 5[b]	101 ± 6
4b	73 ± 6[b]	98 ± 6	108 ± 7	96 ± 6	52 ± 6[b]	110 ± 7
7	117 ± 6	98 ± 6	103 ± 4	99 ± 7	115 ± 8	97 ± 7
Control	100 ± 7[i]	100 ± 6[j]	100 ± 6[k]	100 ± 7[l]	100 ± 6[m]	100 ± 8[n]
Aorta						
3a	89 ± 6[b]	82 ± 5[b]	91 ± 8	101 ± 7	96 ± 5	106 ± 6
4a	104 ± 6	64 ± 5[b]	83 ± 6[b]	143 ± 5[b]	114 ± 7	95 ± 6
4b	50 ± 5[b]	60 ± 6[b]	93 ± 7	68 ± 5[b]	89 ± 7	67 ± 7[b]
7	61 ± 5[b]	75 ± 6[b]	102 ± 6	93 ± 6	107 ± 7	89 ± 6
Control	100 ± 6[o]	100 ± 6[p]	100 ± 7[q]	100 ± 6[r]	100 ± 6[s]	100 ± 5[t]
Feces						
3a	277 ± 9[b]	202 ± 80[b]	194 ± 17[b]	99 ± 6	139 ± 8[b]	133 ± 14[b]
4a	117 ± 7	94 ± 5	98 ± 6	69 ± 5[b]	97 ± 4	101 ± 5
4b	137 ± 8[b]	146 ± 6	65 ± 6[b]	108 ± 7	103 ± 6	118 ± 6
7	100 ± 9	113 ± 7	74 ± 5[b]	112 ± 6	115 ± 5	85 ± 4
Control	100 ± 6[u]	100 ± 6[v]	100 ± 7[w]	100 ± 6[x]	100 ± 8[y]	100 ± 6[z]

[a] Food consumption: control 18.79 g/d, **3a** 18.02 g/d, **4a** 18.57 g/d, **4b** 16.19 g/d, **7** 19.07 g/d. All neutral lipids are represented except triglyceride and cholesterol esters. For compound doses, see Table 3.

[b] $P \leq 0.001$.

[c] 58.15 mg lipid/gram wet tissue.

[d] 9.18 mg cholesterol/gram wet tissue.

[e] 6.37 mg triglyceride/gram wet tissue.

[f] 15.70 mg/neutral lipid/gram wet tissue.

[g] 27.14 mg phospholipid/gram wet tissue.

[h] 12.02 mg protein/gram wet tissue.

[i] 68.20 mg lipid/gram wet tissue.

[j] 12.02 mg cholesterol/gram wet tissue.

[k] 11.20 mg triglyceride/gram wet tissue.

[l] 16.98 mg neutral lipid/gram wet tissue.

[m] 20.06 mg phospholipid/gram wet tissue.

[n] 42 mg protein/gram wet tissue.

[o] 67.5 mg lipid/gram wet tissue.

[p] 5.77 mg cholesterol/gram wet tissue.

[q] 9.85 mg triglyceride/gram wet tissue.

[r] 15.28 mg neutral lipid/gram wet tissue.

[s] 28.8 mg phospholipid/gram wet tissue.

[t] 11.71 mg protein/gram wet tissue.

[u] 11.58 mg of lipid/gram wet tissue.

[v] 2.84 mg of cholesterol/gram wet tissue.

[w] 1.86 mg of triglyceride/gram wet tissue.

[x] 3.39 mg of neutral lipid/gram wet tissue.

[y] 5.70 mg phospholipid/gram wet tissue.

[z] 6.99 mg protein/gram wet tissue.

chylomicron cholesterol levels. Compounds **3a, 4a, 4b** and **7** reduced VLDL cholesterol levels by 29–73% and LDL cholesterol levels by 13–33%; nevertheless, HDL cholesterol levels were elevated by 13% by **4a** at 10 mg/kg/d and 240–309% by **3a** and **4b** at 20 mg/kg/d. Compound **7** increased HDL cholesterol levels by 52%. Triglyceride contents were reduced in the VLDL and LDL by **3a**. Neutral lipids were reduced in the VLDL by **4a, 4b** and **7**, in LDL by **3a** and in HDL by **3a**. Compound **7** caused a significant increase in HDL triglyceride levels. Chylomicron phospholipids were reduced by **4b** and **7**. VLDL and HDL phospholipids were reduced by **3a, 4a** and **4b** and LDL phospholipid levels by **3a**. VLDL protein content was reduced by **3a, 4b** and **7**, and LDL protein content was lowered by **3a**; nevertheless, **4a** and **7** caused elevations in LDL protein content. Electrophoresis of LDL and HDL apoproteins demonstrated that the drug treatment did not affect the apoprotein content of LDL. Only apo-B was present at what appeared to be normal concentrations. In the HDL fraction, apo-E and apo-A I bands were more dense suggesting a higher quantity of these apoproteins was induced by drug treatment.

Hepatic mouse enzyme activities were reduced by the drugs, *e.g.*, cytoplasmic acetyl-CoA synthetase activity was reduced in a concentration-dependent manner by **3a, 4a, 4b** and **7** (Table 6). HMG-CoA reductase activity was reduced in a concentration-dependent manner by **3a** and **4b** but was elevated by **7**. Acyl-CoA cholesterol acyltransferase activity was reduced by all the compounds in a concentration-dependent manner. Cholesterol ester hydrolase activity was elevated by **3a** but was reduced by **4a** and **7**. Cholesterol 7α-hydroxylase activity was inhibited by **4b** and by **7** at 100 μM. Acetyl-CoA carboxylase activity was inhibited by **4b** only at all concentrations. Compound **3a** was effective at 100 μM. *sn*-Glycerol-3-phosphate acyltransferase and phosphatidylate phosphohydrolase were

TABLE 5

The Effects of Hypolipidemic Agents on Serum Lipoprotein of Sprague-Dawley Male Rats at 10 or 20 mg/kg/d Orally for 14 d

	Control (%)				
N = 6	Cholesterol	Triglyceride	Neutral lipids	Phospholipids	Protein
Chylomicron					
3a	48 ± 5[a]	91 ± 7	73 ± 6[a]	102 ± 6	102 ± 6
4a	126 ± 6[a]	118 ± 6	100 ± 7	130 ± 7	100 ± 6
4b	76 ± 5[a]	103 ± 7	98 ± 6	34 ± 6[a]	93 ± 7
7	116 ± 7	104 ± 6	111 ± 5	74 ± 6	100 ± 6
Control	100 ± 7[b]	100 ± 7[c]	100 ± 8[d]	100 ± 7[e]	100 ± 6[f]
Very low density lipoprotein					
3a	71 ± 6[a]	105 ± 7	98 ± 8	45 ± 5[a]	75 ± 5[a]
4a	27 ± 3[a]	57 ± 4[a]	74 ± 5[a]	70 ± 5[a]	96 ± 5
4b	50 ± 4[a]	80 ± 6[a]	80 ± 6[a]	55 ± 6[a]	42 ± 6[a]
7	59 ± 3	77 ± 5	89 ± 6	103 ± 7	43 ± 6
Control	100 ± 6[g]	100 ± 5[h]	100 ± 7[i]	100 ± 6[j]	100 ± 7[k]
Low density lipoprotein					
3a	67 ± 6[a]	94 ± 6	73 ± 7[a]	61 ± 7[a]	36 ± 6[a]
4a	78 ± 5[a]	70 ± 5[a]	131 ± 6[a]	192 ± 8[a]	145 ± 5
4b	87 ± 5[a]	110 ± 7	129 ± 7[a]	104 ± 7	99 ± 7
7	83 ± 5	91 ± 5	109 ± 7	128 ± 6	180 ± 15
Control	100 ± 5[l]	100 ± 6[m]	100 ± 7[n]	100 ± 6[o]	100 ± 5[p]
High density lipoprotein					
3a	340 ± 8[a]	109 ± 7	119 ± 9	40 ± 5[a]	106 ± 7
4a	113 ± 6	93 ± 7	45 ± 6[a]	38 ± 5[a]	100 ± 6
4b	409 ± 9[a]	95 ± 6	89 ± 7	50 ± 6[a]	110 ± 8
7	152 ± 6	263 ± 6	85 ± 5	142 ± 7	100 ± 6
Control	100 ± 6[q]	100 ± 6[r]	100 ± 7[s]	100 ± 8[t]	100 ± 5[u]

[a] $P \leq 0.001$.
[b] 337 μg/mL.
[c] 67 μg/mL.
[d] 420 μg/mL
[e] 149 μg/mL.
[f] 184 μg/mL.
[g] 190 μg/mL.
[h] 98 μg/mL.
[i] 221 μg/mL.
[j] 26 μg/mL.
[k] 50 μg/mL.
[l] 210μg/mL.
[m] 10 μg/mL.
[n] 45 Mg/mL.
[o] 41 μg/mL.
[p] 122 μg/mL.
[q] 544 μg/mL.
[r] 27 μg/mL.
[s] 620 μg/mL.
[t] 153 μg/mL.
[u] 657 μg/mL.

inhibited significantly by all four drugs in a concentration-dependent manner. Hepatic lipoprotein lipase activity was inhibited by **4b**, the only drug tested.

DISCUSSION

Selected derivatives of the 6-alkoxycarbonyl-3-aryl-1,3,5-triazabicyclo[3.1.0]hexane-2,4-diones and 2-alkoxycarbonyl-5-aryl-1,3,5-triazine-4,6(1H,5H)-diones proved to have effective hypolipidemic activity in rodents when administered either orally or intraperitoneally from 10–20 mg/kg/d. The drugs lowered both serum cholesterol and triglyceride levels effectively and were more effective than a number of commercially available agents. Selectivity derivatives were markedly effective in diet-induced hyperlipidemic mice. The effectiveness of the agents in lowering serum lipid levels was not due to a lower caloric intake. The compounds did cause an increase in lipid excretion by the fecal route. With the exception of **4a**, tissue lipids, *e.g.*, cholesterol and triglyceride, were generally reduced by the agents. Thus, the derivatives did not remove lipids from the blood and redistribute the lipids to the tissues.

The serum lipoprotein fractions appeared to be modulated in an effective manner, reducing VLDL and LDL cholesterol levels and elevating HDL cholesterol levels after only two weeks. The cyclic imides, *e.g.*, phthalimide and saccharin, in general, did not raise HDL cholesterol levels by 14 d. 4-Methylphenyl and acetophenone substitutions on the nitrogen atom of the cyclic imides did lead to elevated HDL cholesterol levels at 20 mg/kg/d (35,36). The current derivatives also have bulky substitution on the nitrogen of the ring, *e.g.*, phenyl or 4-chlorophenyl, suggesting that the pharmacoreceptor requires a certain length and bulk for the best fit. Modulation of VLDL cholesterol levels down and HDL cholesterol up by a therapeutic agent in man supposedly protects against myocardial infarction (4,37). The increased contents of apo-E and apo-AI may increase the receptor mediated uptake of HDL by liver receptors resulting in more excretion of cholesterol *via* the bile. These agents also afforded effects on hepatic regulatory lipid metabolizing enzymes. Suppression of cytoplasmic ATP-dependent citrate lyase, mitochondrial citrate exchange or acetyl-CoA synthetase activities reduced the available cytoplasmic acetyl-CoA for fatty acid and cholesterol synthesis. Only **4b** effectively inhibited the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. The agents were more potent in suppressing acyl-CoA cholesterol acyltransferase, thus reducing cholesterol ester formation and storage. There was an indication that the drug caused an increase in bile acids by stimulating cholesterol 7α-hydroxylase activity, the rate-limiting enzyme for cholesterol catabolism to bile acids. Neutral cholesterol ester

TABLE 6

The *in vitro* Effects of the Hypolipidemic Agents 3a, 4a, 4b and 7 on CF_1 Hepatic *de novo* Lipid Regulatory Enzyme Activities

		Control (%)											
		Compound 3a			Compound 4a			Compound 4b			Compound 7		
N = 6	Control	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM	25 μM	50 μM	100 μM
Mitochondrial citrate exchange	100 ± 6[a]	99 ± 6	84 ± 5	79 ± 4[b]	103 ± 7	91 ± 5	84 ± 5[b]	30 ± 3[b]	28 ± 3[b]	4 ± 1[b]	—	—	—
ATP dependent citrate lyase	100 ± 8[c]	82 ± 5	63 ± 6[b]	48 ± 5[b]	118 ± 8	79 ± 7[b]	74 ± 5[b]	131 ± 8[b]	135 ± 9[b]	151 ± 7[b]	153 ± 1	145 ± 9[b]	105 ± 8
Cytoplasmic acetyl-CoA synthetase	100 ± 7[d]	86 ± 7	78 ± 7[b]	66 ± 6[b]	93 ± 6	68 ± 5[b]	49 ± 5[b]	102 ± 6	86 ± 7	71 ± 5	66 ± 6[b]	55 ± 5[b]	45 ± 4[b]
HMG-CoA reductase	100 ± 8[e]	108 ± 9	85 ± 8	76 ± 8[b]	103 ± 8	106 ± 7	103 ± 6	61 ± 5[b]	53 ± 6[b]	50 ± 5[b]	241 ± 6[b]	203 ± 9[b]	123 ± 9
Acyl-CoA cholesterol acyltransferase	100 ± 7[f]	86 ± 6	75 ± 5[b]	64 ± 5[b]	19 ± 5[b]	17 ± 2[b]	12 ± 2[b]	96 ± 6	68 ± 7[b]	70 ± 5[b]	74 ± 7[b]	31 ± 3[b]	29 ± 4[b]
Neutral cholesterol hydrolase	100 ± 9[g]	112 ± 1	118 ± 9	115 ± 7	74 ± 5[b]	68 ± 6[b]	62 ± 5[b]	—	—	—	129 ± 8[b]	70 ± 6	66 ± 6[b]
Cholesterol 7α-hydroxylase	100 ± 6[h]	99 ± 5	87 ± 4	82 ± 6[b]	102 ± 8	93 ± 7	77 ± 6	50 ± 4[b]	38 ± 4[b]	38 ± 3[b]	96 ± 6	89 ± 7	77 ± 7[b]
Acetyl-CoA carboxylase	100 ± 7[i]	94 ± 7	88 ± 5	79 ± 5[b]	114 ± 8	99 ± 9[b]	98 ± 6	69 ± 7[b]	47 ± 5[b]	45 ± 4[b]	109 ± 8	107 ± 7	99 ± 6
sn-Glycerol-3-phosphate acyltransferase	100 ± 5[j]	90 ± 6	84 ± 5	74 ± 7[b]	94 ± 5	77 ± 6[b]	76 ± 5[b]	32 ± 3[b]	28 ± 4[b]	24 ± 4[b]	84 ± 6	83 ± 5[b]	43 ± 6[b]
Phosphatidylate phosphohydrolase	100 ± 7[k]	81 ± 7	65 ± 6	52 ± 6[b]	61 ± 6[b]	59 ± 6[b]	54 ± 5[b]	19 ± 3[b]	13 ± 2[b]	11 ± 2[b]	66 ± 6[b]	59 ± 5[b]	53 ± 5[b]
Heparin-induced lipoprotein lipase	100 ± 7[l]	—	—	—	—	—	—	66 ± 6[b]	51 ± 5[b]	53 ± 4[b]	—	—	—

[a] 30.8% exchange.
[b] $P \leq 0.001$.
[c] 9.2 mg citrate hydrolyzed/gram wet tissues/20 min.
[d] 10.0 mg acetyl-CoA formed/gram wet tissue/20 min.
[e] 103,020 dpm cholesterol formed/gram wet tissue/60 min.
[f] 289,450 dpm/gram microsomal protein/60 min.
[g] 56,436 dpm/mg protein/60 min.
[h] 86,640 dpm/mg microsomal protein/20 min.
[i] 43,000 dpm/g wet tissue/60 min.
[j] 87,620 dpm/g wet tissue/60 min.
[k] Mg Pi released/gram wet tissue/30 min.
[l] 3112 dpm/g wet tissue/60 min.

hydrolase activity was elevated by **3a**. This enzyme regulates the breakdown of cholesterol esters to free cholesterol. If a similar pattern existed in aorta plaque cells in that the cholesterol ester hydrolase activity was elevated and acyl-CoA cholesterol acyltransferase activity was inhibited by the drug, there would be a reduction in cholesterol ester accumulation as well as plaque growth in the aorta.

Inhibition of the present rate-limiting enzyme activities for *de novo* triglyceride synthesis, *i.e.*, by *sn*-glycerol-3-phosphatase acyltransferase and phosphatidylate phosphohydrolase, would significantly reduce triglyceride levels in tissues and in serum after drug treatment.

These studies suggest that 2-ethoxycarbonyl-3-(4-chlorophenyl)-1,3,5-triaze-4,6(1H,5H)-dione **4b** presented the best profile in reducing lipids and modulating serum lipoprotein lipids in favorable directions. Thus, these agents may have future clinical potential after further study.

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Synthesis of Methyl 11,14,17-Eicosatrienoate-3,3,4,4,8,8,9,9-d_8

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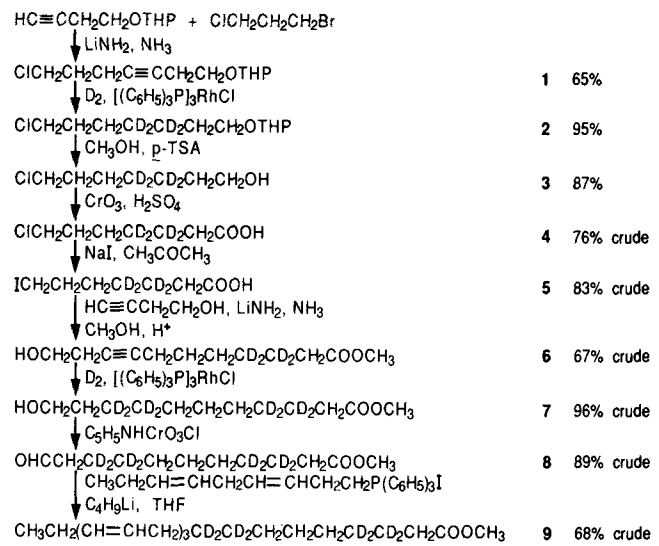
For use in metabolic studies, methyl 11,14,17-eicosatrienoate-3,3,4,4,8,8,9,9-d_8 was prepared by Wittig coupling of 3,6-nonadienyltriphenylphosphonium iodide with methyl 11-oxoundecanoate-3,3,4,4,8,8,9,9-d_8 in the presence of butyl lithium. The *all cis* isomer was separated by high-pressure liquid chromatography using a reverse-phase column. An unexpected by-product, methyl 10,13,16-nonadecatrienoate-2,2,3,3,7,7,8,8-d_8, was also isolated. The structures and the configurations of the compounds were confirmed by nuclear magnetic resonance spectroscopy and mass spectrometry.
Lipids 28, 231–234 (1993).

In continuation of our studies on the metabolism of fats in humans (1,2) and animals, we have synthesized methyl *cis*-11,*cis*-14,*cis*-17-eiocosatrienoate-3,3,4,4,8,8,9,9-d_8 (20:3-d_8). This trienoate has recently been shown to be an intermediate in the conversion of linolenate (9,12,15-18:3) to eicosapentaenoate (5,8,11,14,17-20:5) *via* a nonclassical pathway (3) that utilizes Δ8 desaturation rather than the normal Δ6 desaturation to synthesize 20:5. The deuterated analog has been synthesized to investigate the contribution of this nonclassical pathway to the conversion of 18:3 to 20:5 and the retroconversion of 20:3 to 18:3.

RESULTS AND DISCUSSION

The synthetic steps which we followed are outlined in Scheme 1. The overall approach is similar to the sequence we used to synthesize tetradeuterated 11,14,17-20:3 (4).



SCHEME 1

Abbreviations: EE, diethyl ether; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; PE, petroleum ether (35-60°C); THF, tetrahydrofuran; THP, tetrahydropyranyl.

The reaction sequence involves acetylenic couplings, catalytic deuterations, oxidations and a Wittig reaction.

The tetrahydropyranyl ether of 3-butynol was coupled with 1-bromo-3-chloropropane in the presence of lithium amide in liquid ammonia to give 1-(2-tetrahydropyranyloxy)-7-chloro-3-heptyne, 1, in 65% yield. Catalytic deuteration of 1 with deuterium gas in the presence of Wilkinson's catalyst, *tris*(triphenylphosphine)chlororhodium, gave the tetradeuterated product, 2, in 95% yield. The tetrahydropyranyl group was removed by reaction with methanol and *p*-toluenesulfonic acid to give 7-chloroheptanol-d_4, 3, in 87% yield. Addition of chromium trioxide in 10 N sulfuric acid to compound 3 or rapid addition of compound 3 to the oxidizing medium resulted in a low yield of the desired acid and a high yield of octadeuterated 7-chloroheptyl 7-chloroheptanoate. A 76% yield of the desired acid, 4, together with only a small amount of the ester was obtained by introducing a dilute solution of 3 in acetone under the surface of the rapidly stirred oxidizing medium by means of a syringe pump. If the addition is rapid or the solution is not dilute, the aldehyde formed initially in the oxidation reaction reacts with neighboring alcohol molecules to give a hemiacetal which is then oxidized to the ester (5). If a substantial amount of ester is inadvertently obtained, the acid and alcohol may be recovered. Hydrolysis with alcoholic KOH results in partial replacement of the chlorine by OC_2H_5. Interesterification with 96% formic acid and methanesulfonic acid (6) releases the tetradeuterated 7-chloroheptanoic acid and leaves the tetradeuterated chloroalcohol as the formate ester. The free alcohol may be obtained by interesterification with methanol and mineral acid.

The deuterated chloroacid, 4, was converted to the deuterated iodoacid, 5, in 83% crude yield by reaction with sodium iodide in acetone. Acetylenic coupling of 5 with 3-butynol in the presence of lithium amide in liquid ammonia (7) gave, after esterification, methyl 11-hydroxy-8-undecynoate-3,3,4,4-d_4, 6, in 67% crude yield. Compound 6 was reduced to the octadeuterated product with deuterium in the presence of Wilkinson's catalyst in 96% yield. The saturated hydroxyester 7 was oxidized to methyl 11-oxoundecanoate-3,3,4,4,8,8,9,9-d_8, 8, in 89% crude yield using pyridinium chlorochromate. Compound 8 was then coupled by a Wittig reaction with 3,6-nonadienyltriphenylphosphonium iodide (8,9) to give methyl 11,14,17-20:3-d_8 in 68% crude yield. Reverse-phase high-pressure liquid chromatography was used to isolate a 19% yield (based on the methyl 11-oxoundecanoate-d_8 used) of the all *cis*-20:3-d_8 and a 14% yield of a mixture of the mono-trans and *all cis* isomers. In addition, we obtained a 6% yield of a compound we identified by nuclear magnetic resonance (NMR) as methyl 10,13,16-nonadecatrienoate-2,2,3,3,7,7,8,8-d_8. We were also able to identify a very small amount of methyl octadecatrienoate-d_6 by gas chromatography/mass spectrometry (GC/MS).

Mention is made in the literature (10) of the fact that chromic acid oxidation of alcohols to acids gives poor yields due to the formation of shorter chain acids. The only detailed study reported was by Conant and Aston

(11) who demonstrated that oxidation of butyraldehyde by chromic acid yields some propanoic acid and carbon dioxide. Our analysis of the products formed by the oxidation of 7-chloroheptanol-d_4 with CrO_3 in H_2SO_4 showed the presence of 7-chloroheptanoic acid-d_4, 6-chlorohexanoic acid-d_4 and 5-chloropentanoic acid-d_2 in a ratio of 81:16:3. (We confirmed these observations by the oxidation of octanol which gave three acids and three esters. The esters were the octyl esters of octanoic, heptanoic and hexanoic acids.) Substitution of 6-chlorohexanoic acid-d_4 or 5-chloropentanoic acid-d_2 for 7-chloroheptanoic acid-d_4 in the reaction sequence would yield the 19:3-d_8 and 18:3-d_6 compounds obtained.

The structures and configurations of the final products and of the intermediates were confirmed by nuclear magnetic resonance spectroscopy. The signals for the carbons bearing deuterium atoms were essentially absent, and the chemical shifts for the allylic carbon atoms were consistent with an *all cis* configuration.

EXPERIMENTAL PROCEDURES

Reagents. Butyl lithium, dihydropyran, 3-butynol, 1-bromo-3-chloropropane and pyridinium chlorochromate were obtained from Aldrich Chemical Company (Milwaukee, WI); *tris*(triphenylphosphine)chlororhodium was purchased from Strem Chemicals (Newburyport,MA). Silica Gel (60–200 mesh) and Florisil (100–200 mesh) were obtained from J. T. Baker (Jackson, TN), Silica Gel 60 from American Scientific Products (McGaw Park, IL) and SEP PAK Silica cartridges from Waters Chromatography Division, Millipore Corporation (Milford, MA).

Analytic gas chromatography. A 30 m × 0.25 mm SP2340 or SP2330 fused silica capillary column (Supelco, Inc., Bellefonte, PA) was used for analysis of the binary mixtures of geometric isomers formed. For other analyses, a 6 ft × 4 mm column packed with 3% EGSS-X on 100/120 GasChrom Q or a 5 m × 0.53 mm HP-1 column (Hewlett-Packard, Avondale, PA) was employed. To separate the *trans,cis,cis* and *cis,cis,cis* isomers of 11,14,17-20:3-d_8, it was necessary to use a 100 m × 0.25 mm SP2560 capillary column.

^{13}C NMR. ^{13}C NMR spectra were recorded at ambient temperature with a Bruker (Billerica, MA) WM 300 WB pulse Fourier transform spectrometer operating at 75.5 MHz. Typically, 2500 transients were collected on $CDCl_3$ solutions using 5-mm tubes. $CDCl_3$ served as both the internal lock and secondary reference. Sweep widths of 200 ppm and 8 K real data points limited acquisition time to 0.54 s. Chemical shift values reported are within ±1.85 Hz, *i.e.*, ±0.05 ppm. A pulse width of 3 μs (40°) was employed with no delay between pulses. Decoupling power was held to *ca.* 1 Watt to provide adequate broadband decoupling while minimizing sample heating. The signal from carbons bearing two deuterium atoms or one deuterium atom and a double bond was diminished to such an extent that it was usually not detected.

Mass spectroscopy. Mass spectra were recorded on a Finnigan 4500 mass spectrometer (San Jose, CA) using isobutane chemical ionization and data processing of the isotope distribution against standards (12).

Preparation of 1-(2-tetrahydropyranyloxy)-7-chloro-3-heptyne, 1. Liquid ammonia (*ca.* 800 mL) was charged to a 2-L, three-necked flask equipped with a mechanical stirrer and a dry ice cooled condenser and surrounded by Vermiculite. Ferric nitrate (1 g) was added followed by lithium metal (5.6 g, 800 mmol) which was added in small pieces over 25 min. After the mixture turned grey, the tetrahydropyranyl (THP) ether of 3-butynol (115.5 g, 750 mmol) dissolved in tetrahydrofuran (THF) (100 mL) was added over 45 min. Then 1-bromo-3-chloropropane (59.1 g, 375 mmol) dissolved in THF (50 mL) was added over 15 min. Ten hours later stirring was stopped, the Vermiculite was removed and ammonia was allowed to evaporate overnight through a KOH trap. The next day the flask was immersed in a warm water bath and flushed with argon to remove residual ammonia. Water (300 mL) was added and the mixture was extracted with diethyl ether (EE) (4 × 100 mL). The combined EE layers were washed with saturated ammonium chloride solution (100 mL) and saturated sodium chloride solution (100 mL) and dried (Na_2SO_4). Removal of the drying agent and solvent left a product (138.2 g) which was distilled under reduced pressure to give a forerun containing the THP ether of 3-butynol (47.1 g) b.p. 50–75° at 0.5 torr and a main fraction containing **1** (56.1 g, 64.8% of theory, 90% pure by GC on HP-1) b.p. 112–120°C at 0.4 torr. ^{13}C NMR (ppm): C-1, 65.92; C-2, 20.07; C-3, 77.96; C-4, 78.98; C-5, 16.06; C-6, 31.53; C-7, 43.55. THP group: C-2′, 98.55; C-3′, 30.46; C-4′, 19.30; C-5′, 25.34; C-6′, 62.00.

Preparation of 1-(2-tetrahydropyranyloxy)-7-chloroheptane-3,3,4,4-d_4, 2. Compound 1 (53.2 g, 231 mmol) dissolved in benzene (1 L) was treated with deuterium gas in the presence of *tris*(triphenylphosphine)chlororhodium (4.2 g) in the manner previously described (13). The reduction took about 6 h. Benzene was removed on the rotary evaporator, petroleum ether (PE) was added and the mixture was filtered through a plug of glass wool onto a column (30 mm × 50 cm) containing Silica Gel 60 (120 g) in PE. Elution with 100% PE and PE/EE (90:10, vol/vol) gave the title compound, 2, (52 g, 95% of theory, about 95% pure by GC on HP-1). ^{13}C NMR (ppm): C-1, 67.44; C-2, 29.34; C-5, 26.52; C-6, 32.48; C-7, 45.03. THP group: C-2′, 98.78; C-3′, 30.73; C-4′, 19.65; C-5′, 25.45; C-6′, 62.27.

Preparation of 7-chloroheptanol-3,3,4,4-d_4, 3. Compound 2 (52 g, 218 mmol) was stirred overnight in methanol (500 mL) with *p*-toluenesulfonic acid (1 g). Solid sodium carbonate was added and methanol was removed on the rotary evaporator. Saturated sodium carbonate solution (50 mL) was added and the mixture was extracted with EE (4 × 50 mL) and dried (Na_2SO_4). The drying agent and solvent were removed, and the residue was distilled under reduced pressure to give the title compound, 3, (29.2 g, 87% of theory, 90% pure by GC on HP-1) b.p. 84–90°C at 0.4 torr. Deuterium analysis: 0.23% d_0, 1.12% d_1, 7.96% d_3, 88.76% d_4, 1.43% d_7, 0.12% d_{10}, 0.37% d_{11}. D average = 3.95. ^1H NMR ($CDCl_3$): δ 3.60 (*t*, 2H, CH_2OH); δ 3.50 (*t*, 2H, CH_2Cl); δ 1.69–1.79 (*m*, 3H, CH_2CH_2Cl and O*H*); δ 1.52 (*t*, 2H, CH_2CH_2OH); δ 1.39 (*t*, 2H, CH_2CD_2). ^{13}C NMR (ppm): C-1, 62.66; C-2, 32.22; C-5, 26.43; C-6, 32.36; C-7, 44.97.

Preparation of 3,3,4,4-tetradeutero-7-chloroheptanoic acid, 4. Chromium trioxide (24.05 g, 248 mmol) was dissolved in 10 N H_2SO_4 (250 mL) in a 1-L, three-necked flask equipped with a mechanical stirrer, a thermometer and a narrow plastic tube ending just below the surface of the liquid. The reaction vessel was cooled to about 3°C in an ice-salt bath, and a solution of compound **3** (19.05 g,

123 mmol) dissolved in acetone (250 mL) was added slowly, by means of a syringe pump, below the surface of the rapidly stirred solution at approximately 0.5 mL/min. The addition required about nine hours. Acetone was removed on the rotary evaporator, the residue was diluted with water (300 mL) and extracted with EE (5 × 100 mL). The combined EE extracts were washed with saturated NaCl solution (50 mL) and dried (Na_2SO_4). Removal of the drying agent and solvent left a product which was mostly the desired acid contaminated by only a small amount of ester. This residue was dissolved in EE (150 mL) and extracted with 10% NaOH (2 × 50 mL). The basic solution was washed with EE (25 mL) which was combined with the base extracted EE solution. The EE solution was washed with water (25 mL), dried (Na_2SO_4) and the solvent removed to yield impure tetradeutero-7-chloroheptyl tetradeutero-7-chloroheptanoate (1.23 g). The basic solution was acidified to Congo Red with 3 N HCl (*ca.* 180 mL) and the free acid was extracted into EE (4 × 50 mL). The combined EE extracts were washed with saturated sodium chloride solution (25 mL) and dried (Na_2SO_4). Removal of the drying agent and solvent yielded the desired product, **4**, (15.96 g, 76% crude yield, approximately 78% pure by GC on HP-1). An analytical sample was obtained by passage through a Waters SEP PAK silica cartridge with hexane (Milford, MA). ^{1}H NMR ($CDCl_3$): δ 3.51 (*t*, 2H, CH_2Cl); δ 2.33 (*s*, 2H, CH_2COOH); δ 1.73–1.78 (*m*, 2H, CH_2CH_2Cl); δ 1.42 (*t*, 2H, CH_2CH_2CH_2Cl). ^{13}C NMR (ppm): C-1, 180.3; C-2, 33.69; C-5, 26.20; C-6, 32.26; C-7, 44.90.

Preparation of 3,3,4,4-tetradeutero-7-iodoheptanoic acid, 5. Crude compound **4** (13.7 g, 81.3 mmol) was dissolved in acetone (250 mL). Sodium iodide (15.3 g) was added and the mixture was heated at reflux temperature for 60 h. The reaction mixture was cooled and the solvent was removed on the rotary evaporator. The reaction mixture was poured into EE and a precipitate formed. The mixture was washed with water to remove the solid. The EE solution was dried (Na_2SO_4) and the EE was removed to yield the desired product, **5**, (17.55 g, 83% crude yield, *ca.* 75% pure by GC on HP-1). An analytical sample was obtained by passage through a Waters SEP PAK silica cartridge with PE/EE (95:5, vol/vol); m.p. 42–44°C. ^{1}H NMR ($CDCl_3$): δ 3.16 (*t*, 2H, CH_2I); δ 2.32 (*s*, 2H, CH_2COO); δ 1.80 (*m*, 2H, CH_2CH_2I); δ 1.38 (*m*, 2H, CH_2CH_2CH_2I). ^{13}C NMR (ppm): C-1, 180.24; C-2, 33.69; C-5, 29.79; C-6, 33.12; C-7, 6.86.

Preparation of methyl 11-hydroxy-8-undecynoate-3,3,4,4-d_4, 6. Liquid ammonia (*ca.* 500 mL) was charged to a 1-L, three-necked flask equipped with a mechanical stirrer and a dry-ice cooled condenser and surrounded by Vermiculite insulation. Ferric nitrate (1 g) was added followed by slow addition of lithium metal (4.7 g, 673 mmol). After the reaction mixture assumed a grey color, a solution of 3-butynol (23.55 g, 336 mmol) in THF (50 mL) was added dropwise over 30 min. Then compound **5** (17.5 g, 67 mmol) in THF (50 mL) was added over 30 min. Eight hours later the Vermiculite insulation was removed, and ammonia was vented through a KOH trap. The next morning, the reaction vessel was immersed into a warm water bath and argon was passed through the flask to remove residual ammonia. The reaction mixture was cooled, diluted with water (200 mL) and acidified with 5 N H_2SO_4 (200 mL). The reaction mixture was extracted into EE (5 × 50 mL) and the EE solution was washed with saturated ammonium chloride solution (100 mL) and saturated sodium chloride solution (100 mL) and dried (Na_2SO_4). After removal of the drying agent and solvent, excess 3-butynol was removed by distillation under reduced pressure. Methanol (100 mL) and concentrated H_2SO_4 (1 mL) were added to the residue and the solution was heated under reflux for one hour. The reaction mixture was neutralized with solid sodium bicarbonate and solvent was removed on the rotary evaporator. The residue was diluted with water and extracted into EE (2 × 40 mL). The EE solution was dried (Na_2SO_4) and, after removal of the drying agent and solvent, distillation yielded the desired compound, **6** (9.72 g, 67% crude yield, about 85% pure by GC on HP-1) b.p. 112–120°C at 0.35 torr. ^{1}H NMR ($CDCl_3$): δ 3.58–3.62 (*m*, 5H, OCH_3, CH_2OH); δ 2.33–2.37 (*m*, 3H, CH_2CH_2OH, O*H*); δ 2.22 (*s*, 2H, CH_2COO); δ 2.06–2.11 (*m*, CH_2C=C); δ 1.30–1.44 (*m*, 4H, $CD_2CH_2CH_2$). ^{13}C NMR (ppm): C-1, 174.13; C-2, 33.62; C-5,6, 28.00,28.52; C-7, 18.51; C-8, 82.05; C-9, 76.44; C-10, 22.98; C-11, 61.22; OCH_3, 51.31.

Preparation of methyl 11-hydroxyundecanoate-3,3,4,4,8,8,9,9-d_8, 7. Compound **6** (3.63 g, 16.8 mmol) in benzene (100 mL) was treated with deuterium gas in the presence of *tris*(triphenylphosphine)chlororhodium as previously described (13). Benzene was removed on the rotary evaporator and the red residue was diluted with PE. The mixture was filtered onto a column (15 mm × 25 cm) containing Baker silica gel in PE. Elution with mixtures containing up to 75% EE in PE gave the desired product, **7** (3.62 g, 96% yield, 95% pure by GC on HP-1). Deuterium distribution: 0.03% d_0, 0.01% d_1, 0.01% d_2, 0.03% d_3, 0.22% d_4, 4.73% d_7, 92.97% d_8, 1.85% d_9, 0.15% d_{10}, 0.01% d_{11}. D average = 7.96. ^{1}H NMR ($CDCl_3$): δ 3.63 (*s*, 3H, OCH_3); δ 3.58 (*t*, 2H, CH_2OH); δ 2.25 (*s*, 2H, CD_2CH_2C=O); δ 1.51 (*t*, 2H, CH_2CH_2OH); δ 1.24 (*s*, 6H, (CH_2)$_3$). ^{13}C NMR (ppm): C-1, 174.32; C-2, 33.84; C-5,6,7, 28.92-29.21; C-10, 32.49; C-11, 62.93.

Preparation of methyl 11-oxoundecanoate-3,3,4,4,8,8,9,9-d_4, 8. Pyridinium chlorochromate (9.46 g, 43.8 mmol) was slurried in methylene chloride (100 mL) in a 250-mL, three-necked flask equipped with a mechanical stirrer and a thermometer. While a stream of nitrogen was passed through the apparatus, the reaction mixture was cooled to 10°C with an ice bath. Compound **7** (6.7 g, 30.2 mmol) dissolved in methylene chloride (15 mL) was added in one portion. The ice bath was removed, the reaction mixture turned black and the temperature rose to 19°C. Diethyl ether (50 mL) was added 1.5 h later to the tarry material and was decanted after 10 min of stirring. This was repeated twice with fresh portions (50 mL and 30 mL) of EE. The combined EE extracts were passed through a column (1.5 × 25 cm) containing dry Florisil (15 g) and the column was flushed with EE (50 mL). The solvent was removed on the rotary evaporator to yield compound **8**, (6 g, 89% crude yield, 74% pure by GC on HP-1). An analytical sample was obtained by passage through a Waters SEP PAK silica cartridge with hexane. ^{1}H NMR ($CDCl_3$): δ 9.74 (*t*, 1H, C*H*O), δ 3.64 (*s*, 3H, OCH_3), δ 2.38 (*s*, 2H, CH_2CHO), δ 2.25 (*s*, 2H, CD_2CH_2COO), δ 1.25 (*m*, 6H, (CH_2)$_3$). ^{13}C NMR (ppm): C-1, 174.23; C-2, 33.83; C-5,6,7, 29.0-29.7; C-10, 43.66; C-11, 202.78; methyl group, 51.39.

Preparation of methyl 11,14,17-eicosatrienoate-3,3,4,4,8,8,9,9-d_8, 9. Nonadienyltriphenylphosphonium

iodide (8,14) (29.12 g, 56.9 mmol) was slurried in THF (175 mL) in a 500-mL, three-necked flask equipped with a mechanical stirrer, a low temperature thermometer and a $CaCl_2$ drying tube. While a stream of nitrogen was passed through the apparatus immersed in an ice bath, butyl lithium (2.5M, 24 mL, 60 mmol) was added in portions over 10 min. The temperature achieved a maximum of 17°C and a very dark red solution formed. The ice bath was removed and compound 8 (10.98 g, 49.6 mmol) in THF (20 mL) was added in one portion and the temperature rose from 20–30°C. The color of the reaction mixture became a little lighter. About one hour later, saturated sodium chloride solution was added, and the organic layer was separated and dried (Na_2SO_4). Removal of the drying agent and solvent left a solid and a liquid (38.8 g). This was diluted with PE and filtered onto a column (22 mm × 35 cm) packed with Baker silica gel (40 g) in PE. Elution with PE and PE/EE (95:5, vol/vol) gave the desired compound, 9 (11.1 g, 68% of theory, 69% pure by GC on SP2330). Principal contaminant was 19:3-d_8.

The *all cis* isomer was separated by reverse phase chromatography on an HPLC column (Serva Feinbiochemica, Heidelberg, Germany) (5 × 25 cm) 5 micron Octadecyl Si 100 Polyol using 10% water in acetonitrile as eluant. From 9.9 g of reaction mixture, separated in approximately 1.4 g batches, there was obtained, after pooling similar fractions, 0.93 g of methyl nonadecatrienoate-d_8, 3.07 g of methyl *cis*-11, *cis*-14,*cis*-17-eicosatrienoate-3,3,4,4,7,7,8,8-d_8 and 2.28 g of a mixture of the mono-trans and *all cis* 20:3-d_8 isomers. The overall yield of 20:3-d_8 in the Wittig reaction was 33% and for the overall reaction sequence it was 6.4%.

Deuterium distribution: 0.01% d_0, 0.01% d_1, 0.02% d_2, 0.01% d_3, 0.09% d_4, 0.10% d_5, 4.98% d_7, 94.16% d_8, 0.48% d_9, 0.11% d_{10}, 0.02% d_{13}. D average = 7.95. ^{1}H NMR ($CDCl_3$): δ 5.28–5.42 (*m*, 6H, C*H*=C*H*); δ 3.65 (*s*, 3H, OCH_3); δ 2.77 (*t*, 4H, C=CCH_2C=C); δ 2.27 (*s*, 2H, CH_2COO); δ 2.01–2.12 (*m*, 4H, CH_2C=C); δ 1.25 (*m*, 6H, (CH_2)$_3$); δ 0.96 (*t*, 3H, CH_3). ^{13}C NMR (ppm): C-1, 174.3; C-2, 33.9; C-5,6,7, 29.0-29.3; C-10, 27.0; C-11, 130.3; C-12, 127.6; C-13,16, 25.6; C-14,15, 128.2; C-17, 127.1; C-18, 131.9; C-19, 20.5; C-20, 14.3; OCH3, 51.4.

The 19:3-d_8 was identified as methyl 10,13,16-nonadecatrienoate-2,2,3,3,7,7,8,8-d_8 by the fact that its NMR spectrum was identical to that of 20:3-d_8 with the sole exception of the absence of a chemical shift of δ 2.27 for CH_2COO in the ^{1}H NMR spectrum and of 33.9 for C-2 in the ^{13}C NMR.

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METHOD

Antagonism of [^{3}H]Fatty Acid Incorporation into Vimentin by Sodium Pyruvate: Pitfalls of Protein Acylation

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In the course of studying possible fatty acid acylation of vimentin by cultured bovine lens epithelial cells, several potential pitfalls of protein-fatty acid acylation were recognized. Even exhaustive delipidation of vimentin with organic solvents failed to remove all noncovalently associated [^{3}H]palmitate and [^{3}H]myristate. Hydroxylamine treatment of vimentin, separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), failed to remove either palmitate or myristate derived radiolabel. Hydroxylamine treatment did remove palmitate label from a group of lower molecular weight proteins. The myristate radiolabel associated with vimentin recovered after SDS-PAGE and subjected to acid hydrolysis was shown due to incorporated [^{3}H]amino acids, mainly glutamic acid, generated from the fatty acid. Adding excess sodium pyruvate to labeling media has been used by others to reduce the metabolic conversion of fatty acids to amino acids; however, no direct evidence in support of this antagonism was presented. We observed that inclusion of sodium pyruvate at between 5 and 20 mM in the labeling medium produced a dramatic decrease in incorporation of myristic acid radiolabel into vimentin. However, inclusion of even 20 mM pyruvate did not completely antagonize the metabolic conversion of fatty acid label to amino acids. Furthermore, the sodium pyruvate antagonism could be totally obscured if the exposure of X-ray film by fluorography was even slightly prolonged. The results illustrate the danger in assuming that solvent extraction totally delipidates proteins and that adding sodium pyruvate to labeling media prevents the transfer of fatty acid label to amino acids. Caution is necessary to conclude that radiolabel associated with specific proteins following incubation of cells with labeled fatty acid is due to covalent attachment of the fatty acid to the protein.
Lipids 28, 235–240 (1993).

The present study reexamines potential pitfalls of recognizing fatty acid acylation of cellular proteins. This could be worthwhile in view of the expanding interest in lipid-modifications of proteins. In the course of studying possible fatty acid acylation of vimentin, incorporation of radiolabel from [^{3}H]palmitic and [^{3}H]myristic acids that initially appeared to be due to covalent acylation of vimentin was ultimately shown to be due to incorporation of [^{3}H]labeled amino acids generated by fatty acid metabolism. Initial evidence that vimentin was covalently acylated by [^{3}H]palmitate and [^{3}H]myristate came from observing that [^{3}H]fatty acid was released from exhaustively delipidated vimentin by hydrolysis and from observing no inhibition by sodium pyruvate of [^{3}H]fatty acid incorporation into vimentin. Extensive extraction of cellular proteins with organic solvent is said to completely remove all noncovalently bound labeled lipid from proteins (1,2), and inclusion of 5 mM sodium pyruvate in the labeling media is said to block metabolic conversion of radiolabel from fatty acids into amino acids (3). We present here the first direct evidence that exhaustive delipidation of protein with organic solvents may not remove all noncovalently associated [^{3}H]fatty acid, and inclusion of even 20 mM sodium pyruvate in the labeling media may only partially block incorporation of [^{3}H]fatty acid into protein *via* amino acids, and even this block might be masked if the time used to detect labeled protein by fluorographic exposure of X-ray film is even slightly prolonged.

Vimentin, the single most abundant protein of bovine lens epithelial cells in culture (results of this study), is a cytoskeletal component which bridges nuclear and plasma membranes (4). The covalent attachment of fatty acid to other cytoskeletal proteins, such as actin (5), ankyrin (6) and vinculin (3), could contribute to their association with cellular membranes.

MATERIALS AND METHODS

Cell culture, labeling conditions and cell fractionation. Bovine lens epithelial cells were cultured and maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% calf serum as described previously in detail (7). At day 4 of subculture, medium on confluent cultures was replaced with serum-free DMEM containing 0.10 mCi/mL of [9,10-^{3}H]palmitic acid (60 Ci/mmol, New England Nuclear Corp., Boston, MA) or [9,10-^{3}H]myristic acid (39 Ci/mmol, New England Nuclear Corp.). In some experiments, the medium was supplemented with 5–20 mM sodium pyruvate. Cells were incubated for 4 h at 37°C in a 5% CO_2/95% air atmosphere.

After incubation, cells were washed four times in palmitate or myristate chase medium (8), twice with physiological buffered saline (PBS), pH = 7.4, scraped into PBS, centrifuged (1000 × *g* for 5 min) and washed once with 4 mL of PBS. The washed cells were hand homogenized in about 1.3 mL of 5 mM tris(hydroxymethyl)-aminomethane (Tris) buffer, pH = 8, containing 1 mM ethylenediaminetetraacetic acid (EDTA) plus 5 mM β-mercaptoethanol (Buffer A) using an Econo-Grind glass-glass mini-homogenizer (Radnoti Glass Tech Inc., Monrovia, CA). Water insoluble proteins were recovered by centrifugation (100,000 × *g* for 1 h, SW 60 rotor). Water soluble proteins were precipitated from the supernatant with 10% trichloroacetic acid (TCA), washed twice with 1% TCA and redissolved in Buffer A. Water insoluble proteins were suspended in about 0.5 mL of 55% (g sucrose/100 g solution) sucrose and overlayed with 0.4 mL of 45, 40, 30, 25 and 8% sucrose. All solutions were in Buffer A. The discontinuous gradient was spun at 100,000 × *g* for 2 h

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Abbreviations: DMEM, Dulbecco's modified Eagle's media; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid; PBS, physiological buffered saline; P_y, pyruvate; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLCK, *N*-α-*p*-tosyl-*l*-lysine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; V, vimentin; V_d, vimentin degradation products.

(SW 60 rotor); major fractions were seen at the 30/40% and 45/55% sucrose interfaces and in the pellet. Fractions were recovered and washed once in Buffer A. All solutions used in cell harvesting, homogenization and cell fractionation contained either no protease inhibitors or *N-α-p*-tosyl-*l*-lysine chloromethyl ketone, (TLCK; 1 mM, Sigma Chemical Co., St. Louis, MO) plus ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N'*,N'-tetraacetic acid (EGTA; 2 mM).

Isolation, recovery and identification of vimentin. Water soluble proteins and proteins from the sucrose gradient were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 12% gels by the Laemmli method (9) and subjected to fluorography using EN^{3}HANCE (New England Nuclear Corp.) according to the manufacturer's instructions. Proteins also were electrotransferred from unstained SDS-PAGE gels to nitrocellulose membranes (Electro-Eluter, Bio-Rad, Richmond, CA), blotted with rabbit antichicken vimentin antiserum (1:1000 dilution) and detected using alkaline phosphatase conjugated goat antirabbit IgG (Sigma Chemical Co.). In some cases, the fixed gels were treated for 18 h with 1 M hydroxylamine at pH 7 or with 1 M Tris, pH 7 (control), prior to staining and fluorography (2). Water soluble proteins were also separated by combined electrophoresis and SDS-PAGE using the mini-Protean II, 2-dimensional cell of Bio-Rad. Proteins were electrotransferred to ProBlott membrane (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and stained and submitted for amino acid compositional analysis and sequence identification by the Edman degradation (Protein Structure Core Facility of the University of Nebraska Medical Center, Omaha, NE).

Vimentin delipidation, alkaline hydrolysis and acid methanolysis. The water insoluble fraction recovered from the 45/55% sucrose interface was extracted with 8 M urea and centrifuged (100,000 × *g* for 1 h). Vimentin is extractable from calf lens by concentrated urea (10). The supernatant was diluted to 1.6 M urea with water and brought to 10% TCA. The protein pellet was washed twice with 1% TCA, lyophilized and delipidated with the following series of organic solvent extractions: five extractions with ice cold chloroform/methanol (2:1, vol/vol), and one each with ice-cold methanol, chloroform/methanol (1:2, vol/vol), chloroform/methanol (1:1, vol/vol), chloroform/methanol/water (1:1:0.3, by vol) and methanol. This is similar to the protein-delipidation procedure used by Iozzo *et al.* (2). Delipidated protein was subjected to SDS-PAGE and fluorography.

Aliquots of the delipidated protein were suspended in 1.0 mL of either 0.67 N KOH in 67% ethanol/water (vol/vol) or 1 mL of 2 N HCl in methanol, heated at 110°C for 24 h or 72 h, respectively, and diluted with 1 mL of water. Unlabeled palmitate and myristate (25 to 100 μg) was added as carrier prior to heating. The alkaline hydrolysate was extracted with three 2-mL aliquots of hexane and the acid methanolysis mixture with three 2-mL aliquots of petroleum ether. Methyl palmitate and methyl myristate recovered from acid methanolysis were separated by thin-layer chromatography as described by James and Olson (8), and the distribution of radiolabel between methyl palmitate and methyl myristate in the excised spots was measured by scintillation counting.

Measurement of amino acid tritium in vimentin. Vimentin was electroeluted as the 59 kDa band excised from SDS-PAGE gels. The eluted protein fraction was dialyzed overnight against 5 mM ammonium bicarbonate containing 0.05% SDS (wt/vol). After adding 50 μg of albumin carrier, the protein suspension was SpeedVac-dried (SpeedVac Concentrator, Savant Inst., Farmingdale, NY), suspended in 50 μL of water and the protein precipitated at −20°C with 450 μL of acetone. The acetone washed pellet was air dried, suspended in 1.0 mL of 6 N HCl and hydrolyzed at 110°C for 18 h *in vacuo*. The hydrolysate was extracted three times with 1 mL of benzene. The aqueous phase was lyophilized, dissolved in a small volume of 10% isopropanol and the amino acids separated by two-dimensional thin-layer chromatography on Whatman No. 1 paper using *n*-butanol/acetic acid/water (1:0.25:0.42, by vol) in the first direction and phenol/water (1:0.25, wt/vol) in the second direction, and visualized by ninhydrin spray (11). The identity of amino acids was determined by comparison with standards. The excised amino acid spots were dissolved in 1 mL of water and the tritium content determined by liquid scintillation counting.

RESULTS

Radiolabeling of vimentin. When cultured bovine lens epithelial cells were homogenized and fractionated in the absence of calcium protease inhibitors, about 50% of the total cellular protein was water soluble and largely accounted for by several polypeptides of about 45–47 kDa (Fig. 1A). Insoluble protein fractions were recovered from a discontinuous sucrose gradient at the 30/40% and 45/55% sucrose interfaces and in a pellet fraction. In the presence of the protease inhibitors (TLCK plus EGTA), the soluble protein recovered from the cells decreased to about 15% of total and the protein recovered from the 45/55% sucrose interface fraction markedly increased, with a particular increase in the 59 kDa polypeptide component of this fraction.

Both the water soluble 45–47 kDa polypeptides and the water insoluble 59 kDa protein of the 45/55% sucrose fraction were labeled by [^{3}H]palmitic and [^{3}H]myristic acids (Fig. 1). The 59 kDa protein was immunostained with vimentin antiserum (Fig. 2). Water soluble polypeptides labeled from [^{3}H]palmitic acid were separated on two-dimensional gels and transferred to a ProBlott membrane (Fig. 3A). The two major components (Unknown 1 and 2) were excised and subjected to amino acid analysis, and Unknown 2 was sequenced. The amino acid composition of both was very similar to bovine vimentin (Fig. 3B), and the sequence of Unknown 2 corresponded to vimentin truncated by 72 amino acids from the amino terminal (Fig. 3C). Thus, the 59 kDa protein of the 45/55% sucrose interface fraction was vimentin and the 45–47 kDa water soluble polypeptides were vimentin degradation products. Nelson and Traub (15) and Ireland and Maisel (16) have shown that vimentin of both ascites tumor cells and lens cortical fiber cells is rapidly degraded by calcium activated proteases which can be inhibited by TLCK and EGTA.

Vimentin, like other cytoskeletal proteins, can be extracted from the cell's water insoluble fraction by 8 M urea (Fig. 4) (10). The urea extracted vimentin was precipitated with trichloroacetic acid after diluting the urea with water. The recovered vimentin was washed with 1% TCA, dried

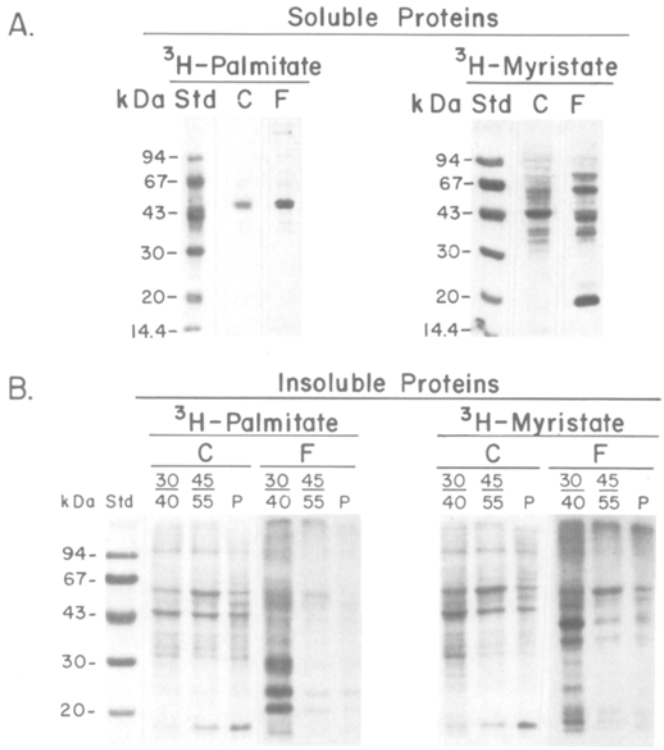


FIG. 1. Labeling of cultured bovine lens epithelial cell proteins with [^{3}H]palmitic acid and [^{3}H]myristic acid. Confluent dishes of cells at day 4 of culture were incubated for 4 h at 37°C in Dulbecco's modified Eagle's media containing 0.1 mCi/mL of either [^{3}H]palmitic or [^{3}H]myristic acid. Cells were separated into water soluble and insoluble fractions. The insoluble fraction was further fractionated on a discontinuous sucrose gradient. A. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography (12–15 d) of soluble proteins. B. SDS-PAGE and fluorography (7 d) of insoluble proteins recovered from the 30/40% and 45/55% sucrose interface and pellet (P) fractions. C, Coomassie blue stained protein. F, fluorography.

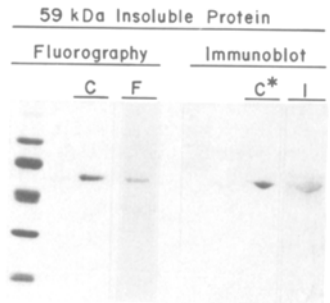


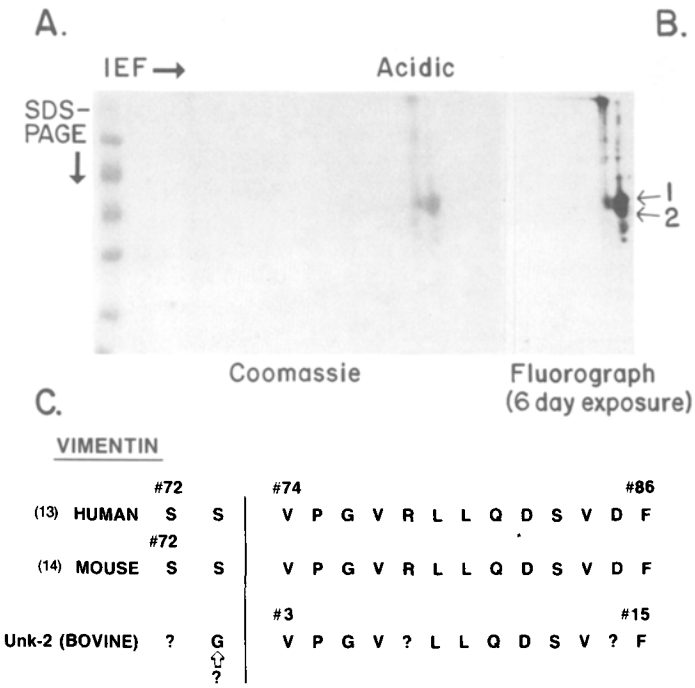
FIG. 2. Fluorography and western blot of the 59 kDa protein from the 45/55% sucrose interface fraction. The 59 kDa band from cells incubated with [^{3}H]palmitic acid was excised from SDS-PAGE gels and rerun in SDS-PAGE and either subjected to fluorography, F, (6 d) or immunostaining, I, with antichicken vimentin antiserum. C*, Coomassie blue staining protein which remained in the gel after transfer. Abbreviations as in Figure 1.

and exhaustively extracted with ice-cold organic solvents as described in Materials and Methods. The tritium content of the [^{3}H]palmitate labeled protein decreased from 35.7×10^6 dpm to 7.02×10^4 dpm (99.8% decrease) and the [^{3}H]myristate labeled protein decreased from 32.7×10^6 dpm to 7.1×10^4 dpm (99.8% decrease). Aliquots of the delipidated protein were subjected to acid methanolysis and alkaline hydrolysis. About 80% of the radiolabel was released from the [^{3}H]palmitate labeled vimentin into the lipid soluble phase following both alkaline hydrolysis and acid methanolysis. About 50% of the radioactivity was released from [^{3}H]myristate labeled vimentin into the lipid soluble phase following both alkaline hydrolysis and acid methanolysis. The release of myristate radioactivity by alkaline hydrolysis was unexpected as myristic acid attaches to protein by amide linkage (7). As discussed below, this observation likely reflects displacement by base of myristate which remained noncovalently associated with vimentin even after exhaustive extraction with organic solvents. Thin-layer chromatography of the recovered fatty acids showed methyl palmitate to account for about 93% of the label from the palmitoylated protein, and methyl myristate for about 73% of that from myristoylated vimentin; the remaining radiolabel comigrated with methyl palmitate.

The release of substantial radiolabel from both [^{3}H]palmitic and [^{3}H]myristic acid labeled vimentin by alkaline hydrolysis sharply contrasted with the apparent lack of effect of hydroxylamine treatment upon release of label from either palmitoylated or myristoylated vimentin separated by SDS-PAGE (Fig. 5). Hydroxylamine did release radioactivity from a group of palmitate labeled proteins between 20 and 30 kDa. This observation indicates that some lens epithelial cell proteins are acylated and that the conditions of hydroxylamine treatment were adequate to completely release fatty acid attached to the protein by an ester linkage.

From these observations, we considered the possibility that the lipid soluble radiolabel recovered following acid and base hydrolysis of delipidated vimentin represented trace amounts of noncovalently associated [^{3}H]fatty acid which was not removed by even exhaustive solvent extraction. We compared the release by acid methanolysis of lipid soluble myristate-radiolabel from vimentin, which was directly recovered from SDS-PAGE gels, with that from vimentin which was recovered by urea extraction and then delipidated. About 50% of the protein radiolabel was recovered in the organic solvent phase following direct acid methanolysis of the delipidated vimentin, but essentially none appeared as lipid-soluble radiolabel following acid-methanolysis of the protein recovered from the SDS-PAGE gel (Table 1).

Because we now suspected that the incorporation of fatty acid radiolabel into vimentin occurred through labeled amino acids generated from the fatty acids, the labeling studies were repeated with inclusion of excess sodium pyruvate in the incubation media. Increased pyruvate (P_y) is presumed to decrease the metabolic conversion of radiolabel from [^{3}H]fatty acids to amino acids by expanding the cellular acetate pool (2,3); however, data documenting this effect are not available. Adding 5 mM sodium P_y to the labeling media (which contains a basal level of 1 mM) produced no obvious decrease in the incorporation of either [^{3}H]palmitic or [^{3}H]myristic acid into vimentin or other cellular proteins (Fig. 6). This observation appeared to support the idea that vimentin was covalently labeled by these fatty acids. However, when the experiment was repeated using higher concentrations of



B.

mol/100 RESIDUES	BOVINE VIMENTIN (12)	Unk 2	Unk 1
Asp	9.5	12.8	14.0
Thr	3.9	4.9	5.1
Ser	5.8	5.7	5.7
Glu	20.9	19.9	19.7
Pro	4.7	2.3	2.2
Gly	6.4	3.3	3.2
Ala	7.6	7.3	6.8
Val	3.3	5.0	5.5
Met	1.5	1.8	2.0
Ile	2.7	4.2	4.2
Leu	11.0	12.9	12.7
Try	2.5	2.6	2.3
Phe	2.2	2.7	2.5
His	2.0	1.2	1.7
Lys	6.7	5.8	5.3
Arg	7.8	7.7	7.1

FIG. 3. Amino acid composition and sequence of [^{3}H]palmitate labeled soluble protein. A. Bovine epithelial cell soluble protein separated by isoelectric focusing and SDS-PAGE. B. Amino acid composition of unknown (Unk) proteins 1 and 2, compared to bovine lens vimentin (ref. 12). C. *N*-terminal amino acid sequence of Unknown 2, compared to human and mouse vimentin (residues 72-86) (Refs. 13 and 14). Abbreviations as in Figure 1.

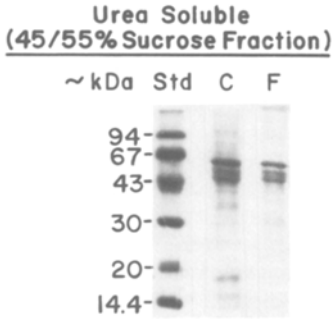


FIG. 4. Urea soluble vimentin. Protein extracted by 8 M urea from the 45/55% sucrose interface fraction of cells incubated with [^{3}H]palmitic acid. F, fluorography, 6 d; V, vimentin; C, Coomassie blue.

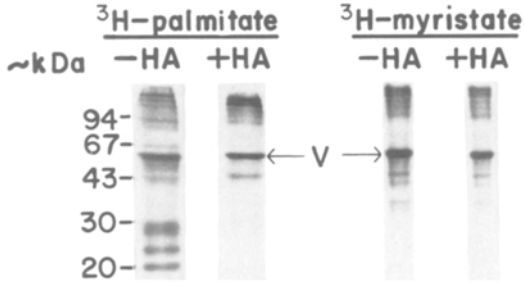


FIG. 5. Effect of hydroxylamine treatment on the release of label from [^{3}H]palmitate and [^{3}H]myristate labeled protein. Insoluble protein recovered from the 45/55% sucrose interface fraction was separated by SDS-PAGE. Gels were soaked in either 1 M Tris, pH 7, (−HA) or in 1 M hydroxylamine, pH 7, (+HA) for 18 h at room temperature prior to staining and fluorography (7 d). V, vimentin; abbreviations as in Figure 1.

sodium pyruvate, near complete inhibition of myristate labeling of vimentin could be observed depending upon the length of the fluorography exposure time. Following a six-day fluorography exposure, 5, 10 and 20 mM sodium pyruvate all produced similar decreases in the intensity of labeling of vimentin with [^{3}H]myristate (Fig. 7). When the fluorography exposure time was decreased to three days, near complete blockage of labeling of vimentin from [^{3}H]myristic acid was seen. Because blockage by sodium pyruvate of metabolic conversion of fatty acids into amino acids is incomplete, failure to initially recognize this antagonism by fluorography was likely due to exposing the X-ray film for too long (Fig. 6).

Final proof that the apparent incorporation of [^{3}H]myristic acid, and likely [^{3}H]palmitic acid, into vimentin was due to amino acids which were then used for protein synthesis was obtained by acid hydrolysis of labeled vimentin and measuring the radioactive content of the resulting amino acids. As seen before, over 90% of the tritium label in vimentin recovered from the SDS-PAGE gel remained in the water soluble phase following acid hydrolysis and solvent extraction. The essential absence of radiolabel in the organic solvent phase shows that the tritium incorporated into vimentin following incubation of the lens epithelial cells with [^{3}H]myristic acid was not due to acylation by fatty acid. Rather, aspartate, glutamate and serine accounted for essentially all of the radiolabel associated with vimentin, with glutamate alone accounting for about 50% of the total (Table 2).

TABLE 1

Recovery of Lipid Soluble Tritium Following Acid Methanolysis of [^{3}H]Myristate Labeled Vimentin from the 45/55% Sucrose Interface Fraction[a]

Fraction following treatment	Percent distribution of radiolabel: PAGE separated (%)	Urea-separated and delipidated (%)
Aqueous layer	95	47
Solvent layer	5	53

[a]Vimentin directly recovered from sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or vimentin extracted by 8 M urea and then exhaustively delipidated was subjected to acid methanolysis.

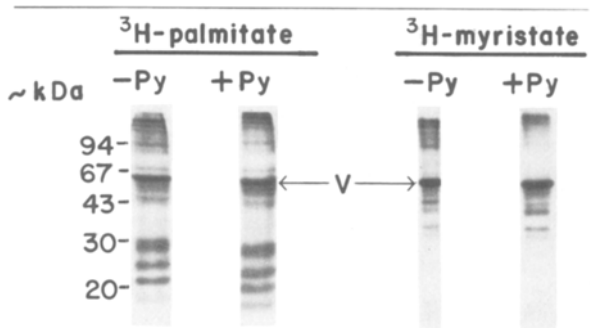


FIG. 6. Effect of sodium pyruvate on labeling of vimentin with [^{3}H]palmitic acid and [^{3}H]myristic acid. Proteins recovered from the 45/55% sucrose fraction of cells in the absence or presence of excess sodium pyruvate (Py, 5 mM) were subjected to fluorography (7 d). V, vimentin.

TABLE 2

Distribution of Radiolabel Among Vimentin's Amino Acids[a]

Amino acid	% Distribution of radiolabel[b]
Ala	<1
Asp	10
Gly	3
Glu	49
His	<1
Leu, Ile	<1
Phen, Try	<1
Ser	38
Thr	<1

[a]Vimentin from cells incubated with [^{3}H]myristic acid was recovered from sodium dodecylsulfate-polyacrylamide gel electrophoresis gels by electroelution. Albumin (50 μg) was added as carrier, and the recovered protein was hydrolyzed in 6N HCl (110°C, 18 h). More than 90% of the radiolabel in the hydrolysate remained in the aqueous phase following extraction with benzene. The aqueous phase was then lyophilized, the residue dissolved in 10% isopropanol and subjected to two-dimensional paper chromatography. Amino acids were visualized by ninhydrin spray and identified by comparison to standards. Spots were excised and counted.

[b]These amino acids accounted for 64% of the total [^{3}H]label theoretically applied to the paper. An additional 13% of the applied radiolabel was found outside of the areas of the identified amino acids.

DISCUSSION

In the process of investigating the possible fatty acid acylation of vimentin, we documented at least two potential pitfalls in detecting fatty acid acylation of cellular proteins. Following even exhaustive organic solvent extrac-

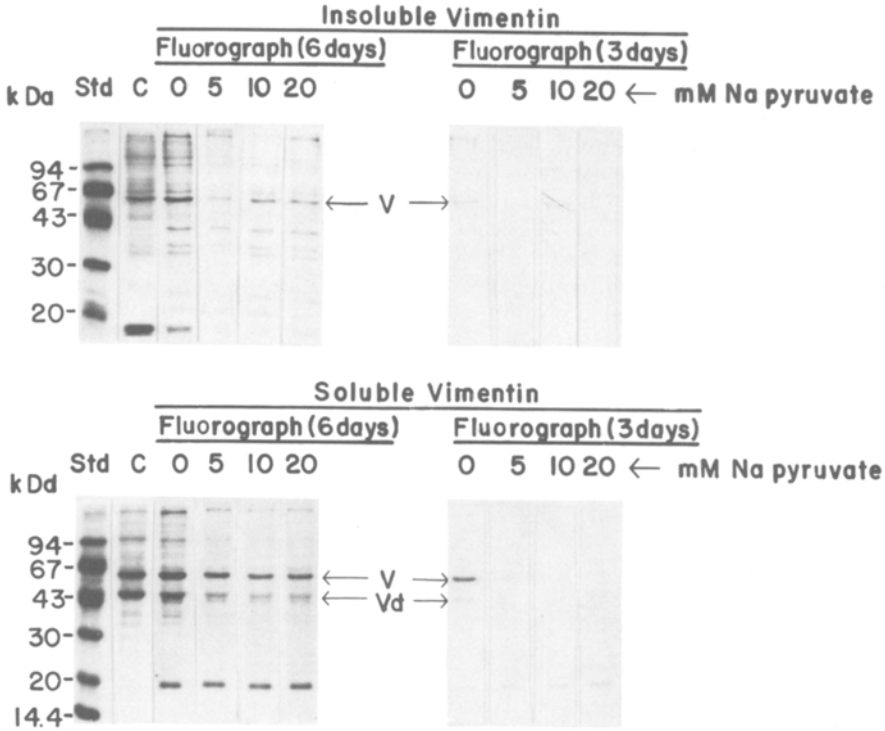


FIG. 7. Antagonism by sodium pyruvate of [^{3}H]myristic acid labeling of soluble and insoluble vimentin. Insoluble vimentin was from the cellular pellet fraction. Cells were incubated with [^{3}H]myristic acid in the absence or presence of excess sodium pyruvate (5, 10 or 20 mM). C, Coomassie stained, V, vimentin, Vd, vimentin degradation products.

tion, [^{3}H]labeled fatty acid can remain noncovalently associated with the protein. In the case of vimentin, 50 to 80% of the radiolabel associated with the protein after delipidation was likely free [^{3}H]palmitic or [^{3}H]myristic acid. By contrast, SDS-PAGE appeared to remove essentially all of noncovalently bound [^{3}H]fatty acid from vimentin. The difficulty in complete delipidation of proteins by solvent extraction is compounded by the high concentration and specific activity of radiolabeled substrates used in the fatty acid acylation of proteins and the low levels of incorporation typically obtained, usually requiring one or more weeks of fluorography exposure for detection. A second potential pitfall involves the use of sodium pyruvate to antagonize the metabolic conversion of fatty acid to amino acid. Because supplementing the labeling media with excess sodium pyruvate does not completely block incorporation of [^{3}H]fatty acid into protein *via* amino acids, careful attention must be given to the fluorography exposure times. Even slightly excessive times can mask the competitive effect of the pyruvate.

Olson *et al.* (1) stated that incorporation of [^{3}H]myristate into 3Ts mouse fibroblasts and PC12 cells occurred mainly due to generation of [^{3}H]amino acids, which were subsequently used for protein synthesis. However, specific proteins were not identified. The present study shows that the labeling of vimentin from either [^{3}H]palmitic or [^{3}H]myristic acid in cultured bovine lens epithelial cells during short-term incubation likely occurred solely by metabolic conversion of fatty acid to amino acids. This route of protein labeling would be greatest for actively synthesized proteins, and vimentin was the single most actively synthesized protein by these cells from [^{3}H]leucine (data not shown). An additional possible complication of protein-fatty acid acylation studies is that protein labeling might occur simultaneously by covalent acylation and amino acid incorporation. In fact, this possibility cannot be excluded in the case of the palmitate radiolabel associated with vimentin, as the SDS-PAGE separation of protein was performed in the presence of reducing agent which might cleave some palmitic acid attached to vimentin by thioester (18). However, retention of palmitic acid label in vimentin after treatment of gels with hydroxylamine supports incorporation of at least some palmitate radiolabel by way of amino acids. Proof of protein-fatty acid acylation can be obtained by showing that base and/or acid hydrolysis of labeled protein recovered from SDS-PAGE gels releases all of the radiolabel from the protein, the released label is soluble in organic solvent and it is shown to be in fatty acid. Recall that SDS-PAGE appeared to remove all noncovalently associated [^{3}H]fatty acid from protein, at least for vimentin.

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Rapid Purification of Rabbit Reticulocyte Lipoxygenase for Electron Paramagnetic Spectroscopy Characterization of the Non-Heme Iron

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An efficient three-step purification technique has been developed for the reticulocyte 15-lipoxygenase from rabbit. Ammonium sulfate fractionated reticulocyte lysate was purified by size exclusion chromatography and preparative scale isoelectric focusing. The entire procedure was complete in less than eight hours and was carried out in batches which typically yielded 10 mg of purified enzyme. The identity and purity of the enzyme were evaluated by *N*-terminal sequencing, sodium dodecylsulfate polyacrylamide gel electrophoresis and specific activity determinations. The enzyme contained approximately one g-atom iron per mole of protein. The iron was present in an electron paramagnetic spectroscopy (EPR) silent, presumably high spin iron(II), form in the isolated enzyme. Treatment with one equivalent of 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid resulted in the appearance of an EPR signal around g6.

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Lipoxygenase occupies a key position in the metabolism of polyunsaturated fatty acids in both plants and animals. The products of lipoxygenase catalysis and subsequent metabolites are potent mediators of an array of biochemical events. For example, there is a substantial body of evidence indicating that rabbit 15-lipoxygenase plays a central role in the maturation of the red blood cell (1). The enzyme apparently catalyzes the peroxidation of mitochondrial lipids resulting in the destruction of the organelle, the inaugural step in the conversion of the reticulocyte into the mature erythrocyte. Like all lipoxygenases, the reticulocyte enzyme contains one non-heme iron atom per molecule of the protein (2). While the reticulocyte enzyme was by a wide margin the first mammalian lipoxygenase to be isolated, the non-heme iron site has not been characterized. By contrast the non-heme iron site of soybean lipoxygenase-1 has been a central focus of studies of that enzyme. Features of the iron have been probed by ultraviolet (UV) visible, electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD), Mössbauer, X-ray absorption, paramagnetic nuclear magnetic resonance (NMR) spectroscopy and magnetic susceptibility measurements (3–5). These experiments demonstrated that the native soybean enzyme contained the metal in an EPR silent high-spin iron(II) form. Treatment of the native enzyme with the product of catalysis converted the metal to a high-spin iron(III) form with a characteristic EPR feature around g6. When the oxidized enzyme was treated with linoleic acid (substrate) in the absence of oxygen, the EPR signal was lost, and a new iron(II) species was detected. Therefore it has been concluded that the unique non-heme iron site plays a fundamental redox role in the catalytic mechanism of the enzyme. The non-heme iron sites in porcine leukocyte 5- and 12-lipoxygenases have been recently investigated and also found to be EPR active (6). However, the leukocyte enzymes as isolated displayed an EPR signal around g5.2 which did not require and was not affeced by the product of catalysis. Also the signal was not abolished by treatment with a reducing agent, nordihydroguaiaretic acid. Two purification procedures for rabbit 15-lipoxygenase have been published (2,7). While the procedures result in the isolation of homogeneous protein in good yields, there has been no apparent progress in the characterization of the non-heme iron site using spectroscopic probes. There is some indication that the purification scheme for the enzyme may need to be tailored to the specific application or investigation. For example, the purification procedure developed first was found not to provide protein samples suitable for crystallization. Initial experiments led us to believe that a critical element in the purification for the examination of the non-heme iron site might be speed. The previous procedures both involved multiple resolution steps with intervening concentration and dialysis manipulations spanning several days. Here we present a rapid purification procedure for the reticulocyte enzyme that provides samples of the enzyme suitable for undertaking the characterization of the non-heme iron site by EPR spectroscopy.

MATERIALS AND METHODS

Reticulocytes were obtained from New Zealand white rabbits injected (1.0–2.0 mL) daily for five days with a 1.2% aqueous solution of *N*-acetylphenylhydrazine adjusted to pH 6.8 using HEPES buffer (1 *M*, pH 7.0). The rabbits were bled daily from day 7 through day 12. The amount of blood collected was between 30 and 50 mL per day and was adjusted to maintain a hematocrit level between 18 and 22% cells.

The supernatant from lysed cells (2) was brought to 55% saturation with ammonium sulfate, and the mixture was centrifuged. The pellet was redissolved in potassium phosphate buffer (0.01 *M*, pH 6.0) and was dialyzed against the same buffer at 5°C. Precipitation was removed by centrifugation, and the supernatant was stored for future use in polypropylene tubes submerged in liquid nitrogen.

Samples (10–15 mL) of the thawed extracts were applied to a Sephacryl S200 HR column (2.5 × 40 cm) equilibrated with Tris buffer, 0.05 *M*, pH 7.2, containing 1 m*M* dithiothreitol (DTT) and 1 mM $MgCl_2$. Elution was carried out with the same buffer at a flow rate of 2 mL min^{-1}. During elution the buffer reservoir was purged continuously with nitrogen and was maintained at 5°C. Fractions (4 mL) were collected and stored under a nitrogen atmosphere on ice during subsequent analysis. Aliquots

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic spectroscopy; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; PVDF, polyvinylidenedifluoride, SDS, sodium dodecylsulfate; UV, ultraviolet.

of the fractions were subjected to sodium dodecylsulfate (SDS) polyacrylamide determinations (8) and measurement of absorbance at 280 and 560 nm.

Pooled fractions (5, 20 mL) from the gel filtration experiment were combined with glycerol (5 mL), 5/7 ampholyte (3.0 mL, Biorad, Richmond, CA), and ice-cold deionized water to give a final volume of 50 mL. The sample was loaded into a preparative scale isoelectric focusing apparatus (Rotofor, Biorad), and a constant power (15 W) was applied for a period of four hours. The fractions were subjected to pH, activity (2) and SDS polyacrylamide gel electrophoresis determinations as well as measurement of absorbance at 280 and 560 nm. Fractions containing lipoxygenase were stored at −20°C without loss of activity for up to two weeks.

Protein sequence determination was carried out on an Applied Biosystems model 477A liquid-pulsed peptide protein sequencer. Samples were analyzed for iron content using flame atomization atomic absorption spectrometry. EPR measurements were made at 9 GHz using a Varian Century Line spectrometer (Palo Alto, CA) equipped with a liquid helium transfer line and a quartz dewar cavity insert. Spectra were recorded at 25 K using microwave power that avoided saturation. Running conditions were set at 5 mW power, 1 mT modulation amplitude, 4 min scan time and 0.128 s time constant. The samples were prepared for analysis by dialysis against Tris buffer, containing 0.1 *M* NaCl and 1 m*M* ethylenediaminetetraacetic acid (EDTA). The oxidized enzyme was obtained by treatment with 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (9) in a 1:1.1 ratio. Final concentration of the samples for EPR was carried out in a centrifugal concentrator (Amicon Centricon 30, Lexington, MA).

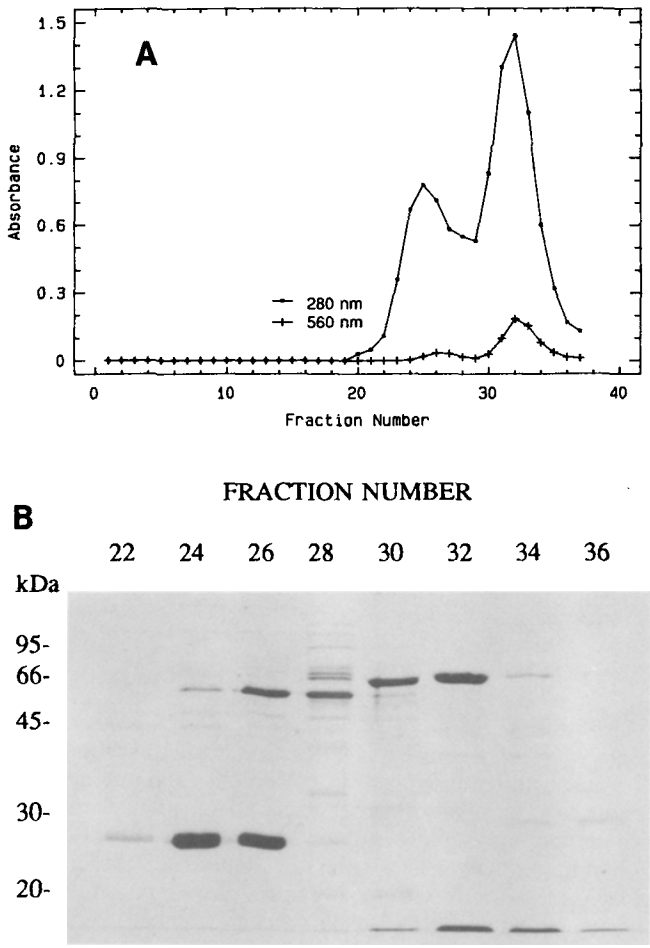

FIG. 1A. Elution profile for size exclusion chromatography of ammonium sulfate fractionated rabbit reticulocyte lysate. Sephacryl S200 HR, Tris HCl (0.05 *M*, pH 7.2, 1 m*M* dithiothreitol, 1 m*M* $MgCl_2$). B. Sodium dodecylsulfate polyacrylamide gel electrophoresis determinations of selected fractions from the size exclusion chromatography.

RESULTS AND DISCUSSION

The mammalian lipoxygenases are susceptible to inactivation with loss of iron in the presence of oxygen (absence of reducing agents) (10,11). Therefore, a rapid purification procedure for rabbit 15-lipoxygenase which avoided exposure to oxygen was investigated. Attempted purification of the enzyme with a chromatofocusing procedure which has been successfully applied to the isolation of the soybean isoenzymes failed. A procedure based on size exclusion chromatography and preparative isoelectric focusing was developed.

The resolution of the proteins obtained from ammonium sulfate precipitated reticulocyte lysate by gel filtration chromatography is shown in Figure 1A. The applied sample was the result of a one-day collection of reticulocytes from two rabbits between day 7 and 14 of the protocol. The collected fractions were monitored for absorbance at 280 and 560 nm which allowed for detection of hemoglobin, a major protein component of the cells. The proteins were resolved between fractions 20 and 40. When fractions in this region were tested for lipoxygenase activity, it was found that the hemoglobin interfered with the assay showing quasi-lipoxygenase activity. SDS polyacrylamide gel electrophoresis (Fig. 1B) showed that a protein of about 75 kDa coeluted with hemoglobin. The only other candidate for the target enzyme in the elution profile was a protein of 70 kDa that was present in fractions 24–28. These samples, however, displayed no lipoxygenase activity. The identity of the 75 kDa protein as 15-lipoxygenase was confirmed by protein sequence analysis. The proteins in the gel were transferred electrophoretically to a polyvinylidenedifluoride (PVDF) membrane. After briefly staining and destaining the membrane containing the transferred proteins, the 75 kDa band was excised and subjected to sequence determination using automated Edman degradation chemistry. A clear amino acid sequence was obtained through 15 Edman cycles which matched exactly with the first 15 amino terminal residues for 15-lipoxygenase deduced from the corresponding c-DNA (12). These observations showed that a simple size exclusion chromatographic experiment was sufficient to provide samples of the enzyme with the only primary contaminant being hemoglobin.

Preparative scale isoelectric focusing was used to resolve the lipoxygenase from the hemoglobin. The result of a typical isoelectric focusing experiment is presented in Figure 2A. The apparatus separated the sample into 20 fractions upon focusing which were monitored for absorbance at 280 and 560 nm. The proteins were clearly resolved into two components one of which displayed

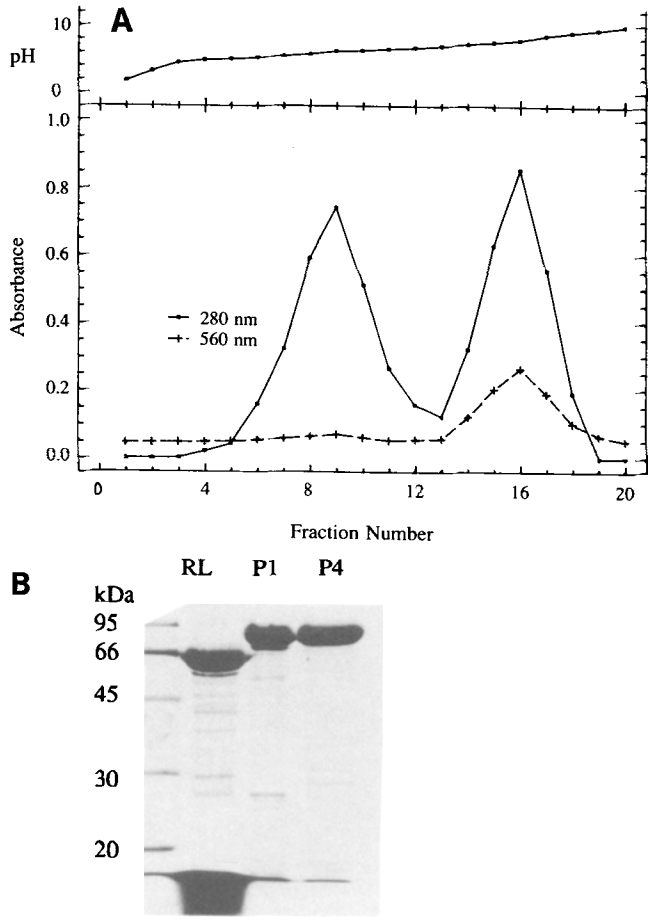

FIG. 2A. Preparative scale isoelectric focusing (pH 5–7) of fractions 30–34 from the size exclusion chromatography. B. Sodium dodecylsulfate polyacrylamide gel electrophoresis determination of fractions 8–10 (RL) from isoelectric focusing. Two isoenzymes of soybean lipoxygenase (P1, P4) were included for comparison.

significant absorbance at 560 nm due to the presence of hemoglobin. The other protein component was the 75 kDa protein previously identified as 15-lipoxygenase. The procedure typically yielded approximately 10 mg of the purified protein. The SDS polyacrylamide gel electrophoresis analysis of samples from fractions containing this protein (Fig. 2B; two isoenzymes of soybean lipoxygenase were run concurrently for comparison) showed the presence of only a single polypeptide and the ampholytes used in the separation procedure. This protein was found in a fraction with a measured pH of 5.5, consistent with the previously reported pI for the 15-lipoxygenase. The turnover number for lipoxygenase catalysis found for the fractions containing the 75 kDa protein was 30 s^{-1}, a value comparable to the reported 25 s^{-1} found previously for highly purified enzyme (2). The purified 15-lipoxygenase was also subjected to analytical isoelectric focusing determination in a polyacrylamide gel from pH 5 to 7 (13). A single band staining for protein and activity at pH 7 was detected in the experiment (data not shown). These experiments demonstrated that useful quantities of 15-lipoxygenase were obtained in an isolation procedure taking less than eight hours.

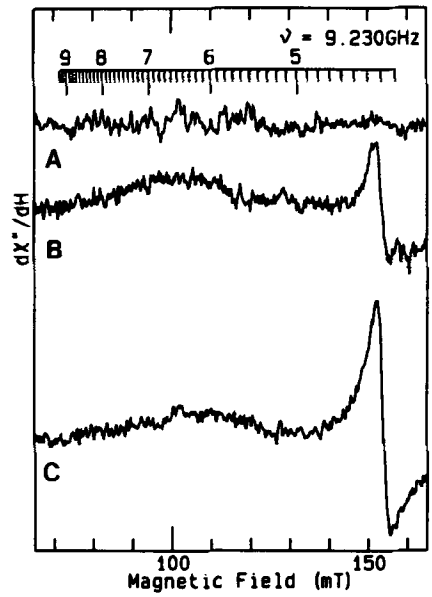

FIG. 3. Electron paramagnetic spectroscopy spectra for samples of purified reticulocyte lipoxygenase, Tris HCl (0.1 *M*, pH 7.4). (A) Spectrum of isolated enzyme, 200 μM. (B) Isolated enzyme, 165 μM, treated with 1.1X 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid. (C) Oxidized enzyme, 200 μM purged with argon and treated with 1.2X linoleic acid.

For further experiments the enzyme solution was dialyzed in nitrogen purged Tris buffer (0.1 *M*, pH 7.4, 1 m*M* EDTA) at 5°C and concentrated in a centrifugal concentrator. These procedures were not accompanied by precipitation of protein. Solutions were obtained with concentrations of the enzyme around 0.20 m*M* displaying undiminished turnover number (30 s^{-1}). Samples of the purified and dialyzed enzyme contained 0.9 g-atom of iron per mole of enzyme using flame atomization atomic absorption spectrometry. The UV-visible spectrum of the purified enzyme contained only the absorbance maximum around 280 nm expected for the contribution of aromatic amino acids. Treatment of the enzyme solution with one equivalent of 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid caused an increase in absorbance in the region 300–350 nm, but no new maximum was obtained. The UV-visible spectroscopy was, therefore, similar to observations on lipoxygenase-1 from soybeans (4). The enzyme as isolated had spectroscopic features consistent with a non-heme iron atom present in a high spin iron(II) form that was oxidized upon treatment with the product of catalysis to give a high spin iron(III) species.

The characterization of the non-heme iron site in the reticulocyte 15-lipoxygenase by EPR spectroscopy is presented in Figure 3. No signals were detected in the solution of the enzyme obtained from the isolation procedure (Trace A). This was consistent with the iron being present in the protein as high spin iron(II) as has been found and confirmed in other spectroscopic measurements for the soybean enzyme. Treatment of the enzyme with 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid resulted in the appearance of two new signals in the EPR spectrum (Trace B), a sharp signal at g4.3 and a broad featureless signal around g6. The integrated intensity of the g6 signal accounted for 15–20% of the iron known to be in the sample.

When samples of the enzyme treated with product were made anaerobic by purging with argon and were subsequently treated with linoleic acid, the spectrum changed (Trace C). There was a diminution in the g6 signal and an increase in the signal at g4.3. The EPR observations were similar in nature to those for the soybean enzyme, but different in degree. The soybean enzyme was converted stoichiometrically into a high spin iron(III) species with the g6 EPR signal by oxidation with product, and the signal was abolished by treatment with substrate in the absence of oxygen (14). We conclude from the similarities in the UV-visible and EPR spectroscopy for the two enzymes that the non-heme iron sites in reticulocyte and soybean 15-lipoxygenase are similar. We speculate that aggregation of the reticulocyte enzyme in the concentrated solutions used in the spectroscopic studies resulted in diminished accessibility of reagents to the active/iron site resulting in incomplete reactions. The development of an efficient precedure for obtaining significant quantities of highly purified rabbit 15-lipoxygenase should facilitate the characterization of the physical and catalytic properties of this important enzyme.

ACKNOWLEDGEMENT

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Simultaneous Determination of the Main Molecular Species of Soybean Phosphatidylcholine or Phosphatidylethanolamine and Their Corresponding Hydroperoxides Obtained by Lipoxygenase Treatment

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A method for the simultaneous determination of the main molecular species of soybean phosphatidylcholine or phosphatidylethanolamine and their corresponding hydroperoxides is described. Hydroperoxides were formed by incubation of phospholipids with lipoxygenase at pH 9.2. Silicic acid column chromatography (silica Sep-Pak column) was used to separate the phospholipids into phosphatidylcholine and phosphatidylethanolamine. A single C-18 reverse-phase column was employed to separate the main molecular species of soybean phosphatidylcholine or phosphatidylethanolamine and their hydroperoxides by high-performance liquid chromatography. The mobile phase consisted of 5% 10 mM ammonium acetate at pH 5 and 95% methanol. The molecular species of phosphatidylcholine and phosphatidylethanolamine were detected at 205 nm; the eluate was mixed with a chemiluminescence reagent (isoluminol and microperoxidase) and monitored by fluorometry. Under the experimental conditions used, three individual molecular species of both soybean phosphatidylethanolamine and phosphatidylcholine (18:3/18:2, 18:2/18:2 and 16:0/18:2), together with their corresponding hydroperoxides, were identified and quantitated.
Lipids 28, 245–249 (1993).

The importance of free radicals in causing alterations in various biological systems has led to numerous attempts to determine lipid peroxides in tissues and biological fluids. The products generated by peroxidizing lipids are exceedingly complex, but considerable progress has been made in defining lipid oxidation at various stages of peroxide degradation. The most commonly used approach is the determination of an end-product of lipid peroxidation, namely malondialdehyde, based on its reaction with thiobarbituric acid. Assay is either by spectrophotometry, as initially described by Yagi (1), or by liquid chromatography (2–4). However, the specificity of this reaction has been questioned (5–7). Other techniques have subsequently been described based on other indices of lipid peroxidation, such as conjugated diene (8) or lipid hydroperoxide measurements (9,10). Progress has also been made in separating phospholipid hydroperoxides by high-performance liquid chromatography (HPLC). In this method, chemiluminescence (CL) detection with isoluminol in the presence of the heme fragment of cytochrome c (11) or with luminol in the presence of cytochrome c is usually used (12,13). Spectrophotometric detection of hydroperoxides, which is based on the formation of an iron thiocyanate complex, has also been described (14).

Several HPLC methods have been reported for separating the molecular species of phospholipids (15–17). However, to our knowledge, the simultaneous determination of phospholipid molecular species and of their hydroperoxides has not been described previously. We report here on an HPLC procedure, which combines the separation of the main molecular species of phosphatidylcholine (PC) or phosphatidylethanolamine (PE) and of their hydroperoxides produced after peroxidation by lipoxygenase. The phospholipid molecular species are detected by means of ultraviolet (UV) spectrophotometry and the hydroperoxides by means of CL. A single sample injection is sufficient for the separation and analysis of PC or PE and their hydroperoxides.

MATERIALS AND METHODS

Chromatographic equipment. Gas-liquid chromatography (GLC) was carried out on a Delsi (Puteaux, France) instrument equipped with a 25-m glass capillary column containing PEG-20 M (Supelco, Bellefonte, PA), and connected to a Delsi integrator. The HPLC system consisted of a Rheodyne injector (Pharmacia, Uppsala, Sweden) with a 50-μL sample loop, a 250 × 4.6 mm RP 18 Spherisorb 5 μm (SFCC/Shandon, Eragny, France) analytical column and a Pharmacia UV/visible light detector. The fluorometer (Spectroflow 980-fluorescence detector, Applied Biosystems, Ramsey, NJ) was equipped with a 5 μL flow cell.

Reagents and chemicals. HPLC-grade solvents and analytical-grade chemicals were obtained from Carlo Erba (Milano, Italy) or from Merck (Darmstadt, Germany). Microperoxidase (MP 11), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), soybean lipoxygenase (type IB, EC 1.13.11.12), soybean PC, soybean PE, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2/18:2 PC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0/18:2 PC) and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (18:0/20:4 PC) and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) were obtained from Sigma (St. Louis, MO).

Phospholipid peroxidation with lipoxygenase. Hydroperoxides were prepared from various molecular species of PE and PC and from soybean PE and PC, either separately or together (50 to 1000 nmol/mL of reaction mixture). Lipoxygenase (500 μg/mL) and 10 mM deoxycholic acid were added to 0.1 M borate buffer, pH 9.2 (18). The mixture (1 mL) was then incubated with gentle shaking at 30°C for 45 min. The reaction was stopped by adding

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Abbreviations: CL, chemiluminescence; GLC, gas-liquid chromatography; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; MTBE, methyl tertiary-butyl ether; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxides; 16:0/18:2 PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; 18:0/20:4 PC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; 18:2/18:2 PC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine; 18:3/18:2 PC, 1-linolenoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PEOOH, phosphatidylethanolamine hydroperoxides; RP, reverse-phase; UV, ultraviolet.

100 μL of 1 M citric acid, and the tubes were placed on ice for 5 min.

Extraction and separation of phospholipids into PC and PE. Phospholipids were extracted with chloroform/methanol (2:1, vol/vol). The mixture was partitioned between the chloroform layer (lower organic phase) and the methanol/water layer (upper phase) by centrifugation at 1500 × *g* for 5 min. The extraction was repeated twice. The lower chloroform layer was collected and evaporated under a stream of nitrogen. The dried total lipids were then dissolved in methanol. For the separation of the soybean PC and PE mixture, the technique recently described by Hamilton and Comai (19) was adapted using Silica-Sep-Pak columns. After elution of neutral lipids with methyl *tertiary*-butyl ether (MTBE)/acetic acid (100:0.2, vol/vol), the polar lipids were separated using 8 mL of MTBE/methanol/10 mM ammonium acetate (10:4:1, by vol) for PE and 10 mL at a ratio of 5:8:2 (by vol) for PC. The various fractions were evaporated to dryness, dissolved in methanol and then each injected into the HPLC system.

Detection and identification of soybean molecular species and hydroperoxides. Separation was achieved by HPLC using an RP18 Spherisorb (250 mm × 4.6, 5 μm) analytical column maintained at 40°C. The mobile phase was composed of 5% 10 mM ammonium acetate, pH 5, and 95% methanol (flow rate, 1 mL/min) filtered through a 0.45 μm HV Millipore filter (Millipore Co., Bedford, MA). The CL reagent was prepared using a slight modification of the technique described by Yamamoto *et al.* (11). Isoluminol (55 mg) was dissolved in a mixture of methanol/borate buffer 0.1 M, pH 9.2, 300:700 (vol/vol); then 10 mg of microperoxidase was added. Under these conditions, the final concentration of isoluminol was 25 mg/L and that of microperoxidase was 5 mg/L. The hydroperoxides were first eluted and then detected in CL reagent at a flow rate of 1 mL/min using a fluorometer as photon detector with the excitation source turned off. The fatty acid molecular species were then eluted and detected at 205 nm. To identify the molecular species after HPLC separation, they were collected and transmethylated at 55°C with 0.5 M methanolic potassium hydroxide and BF_3/methanol (20) and injected into a GLC. Fatty acid methyl ester peaks were identified by their retention times relative to commercial standards (Nu-Chek-Prep, Elysian, MN). On the basis of the findings of Brash *et al.* (21), the intact phospholipids and not the hydrolyzed/reesterified fatty acids are the true substrates of the oxygenation reaction. Consequently, the hydroperoxides produced (specific for PC and PE) were identified using the peroxidation of the standard molecular species of phospholipids.

RESULTS AND DISCUSSION

Separation of phospholipid molecular species and hydroperoxides. Three main individual molecular species (18:3/18:2; 18:2/18:2 and 16:0/18:2) were separated from soybean PC and PE and identified. Each molecular species was quantified by comparing the integrated peak areas with a calibration curve obtained with commercial standards of soybean phospholipids (Fig. 1). The coefficient of variation of this method after extraction and separation was 3.5% for each molecular species (n = 3). The percentage recovery of molecular species was 100% for PE and 93% for PC.

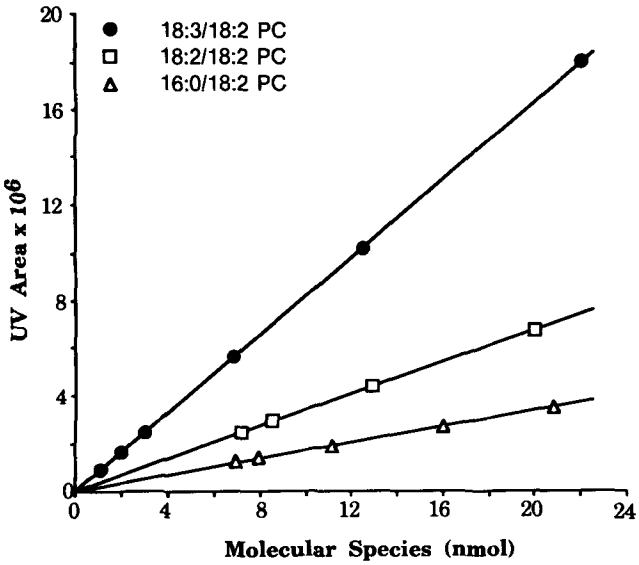

FIG. 1. Calibration curves obtained for soybean phospholipid molecular species detected at 205 nm including 18:3/18:2 PC, 18:2/18:2 PC and 16:0/18:2 PC. The high-performance liquid chromatography conditions used are those described in Materials and Methods. 18:3/18:2 PC, 1-linolenoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; 18:2/18:2 PC, 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine; 16:0/18:2 PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine.

The molecular species were detected at 205 nm both before (Fig. 2A) and after peroxidation (Fig. 2B). There was also a marked decrease in the three molecular species following peroxidation, particularly with 18:3/18:2 and 18:2/18:2. Hydroperoxides were not detected before peroxidation (Fig. 2C). Figure 2D shows the hydroperoxides produced after 45 min of incubation in the presence of lipoxygenase. The products were detected by CL and corresponded to the hydroperoxides derived from 18:3/18:2, 18:2/18:2 and 16:0/18:2, respectively, as identified by comparison with the peroxidation products of the standard molecular species. Each hydroperoxide was quantified on the basis of the relative sensitivity of the CL assay for 15-HPETE. Examples of such calibration curves are shown in Figure 3. A coefficient which took into account the sensitivity of detection of the various hydroperoxides relative to 15-HPETE was applied to determine the concentration of each molecular species (22). The sum of two hydroperoxide species of 18:2/18:2, corresponding to mono and bis hydroperoxides, was taken into account in expressing the results (23). Under these conditions, the correction coefficient was 0.8 for 18:3/18:2, 1.0 for 18:0/20:4, 1.0 for 18:2/18:2, and 3.5 for 16:0/18:2. The coefficient of variation for the hydroperoxide of each molecular species after extraction and separation was 9.8% for three determinations. The percentage recovery of the peroxidized molecular species was 93% ± 5 for PE and 80% ± 7 for PC.

The limit of hydroperoxide detection was 30 pmol when the CL signal was recorded at 0.01 μA and the supply voltage for the photomultiplier was 50% of full range (2000 V). Under our usual experimental conditions, 40% of full range was used to sustain a low background noise; under this condition the limit of detection was 100 pmol.

Optimization of the analytical procedure. The lipoxygenase assay was carried out as described above, at 30°C

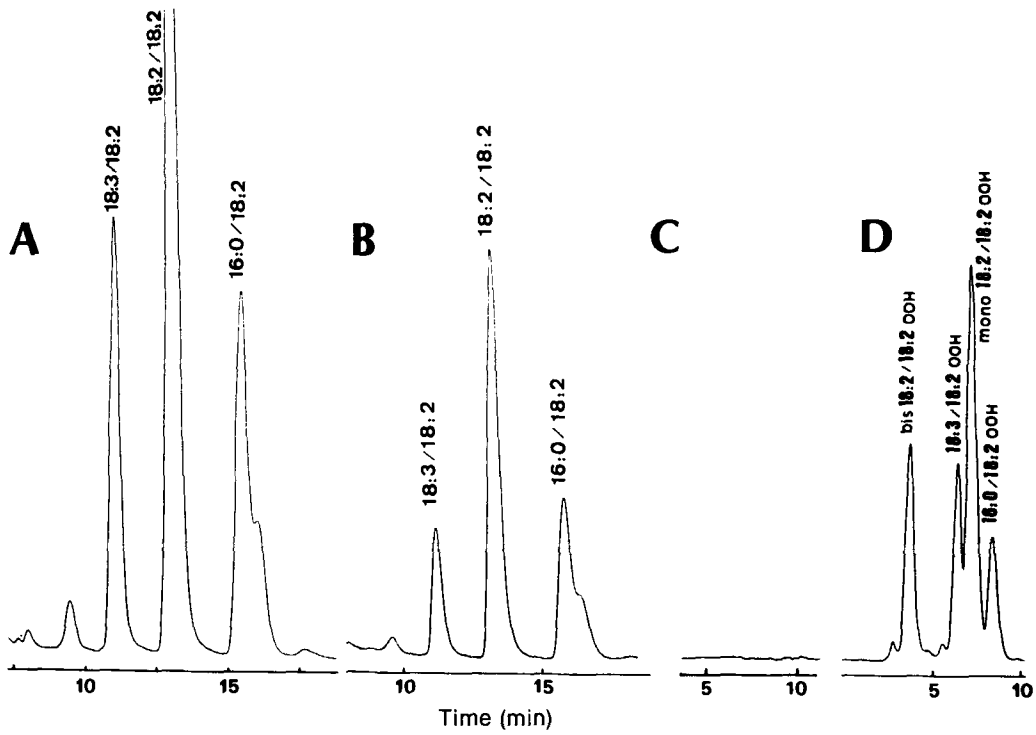

FIG. 2. Separation of molecular species of soybean phosphatidylethanolamine on an analytical RP18 Spherisorb column at 40°C. The mobile phase was methanol/0.01M ammonium acetate (95:5, vol/vol; pH 5.0) delivered isocratically at 1.0 mL/min. Fatty acid molecular species were detected at 205 nm, and the corresponding hydroperoxides were detected using the chemiluminescence reagent (isoluminol and microperoxidase; flow rate 1 mL/min). A, detection at 205 nm before peroxidation; B, detection at 205 nm after 45 min of incubation with lipoxygenase; C, chemiluminescence detection of hydroperoxides (PEOOH) before peroxidation; D, chemiluminescence detection of hydroperoxides (PEOOH) after 45 min of incubation with lipoxygenase.

in the presence of 340 nmol/mL of 18:2/18:2 PC or 18:0/20:4 PC, within a time range from 5 min and 120 min (Fig. 4). A slight decrease in hydroperoxide formation from 18:2/18:2 PC was observed after 45 min of incubation, whereas with 18:0/20:4 PC there was an increase in hydroperoxide formation up to 90 min and a drastic decrease thereafter. This was probably due to the degradation of hydroperoxide with longer incubation times. Thus, 45 min was selected as the incubation time in all the experiments.

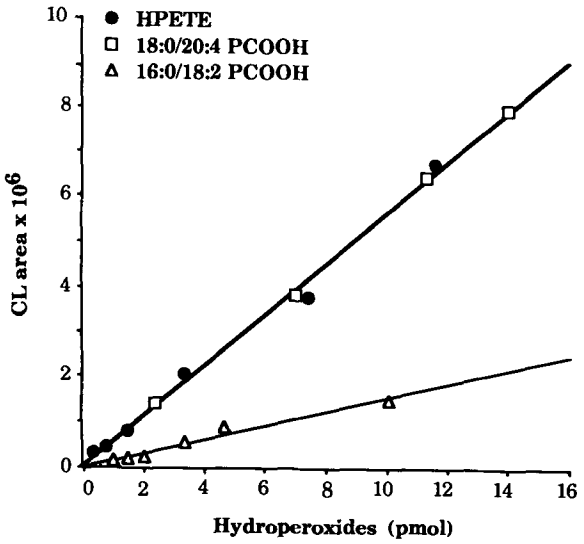

FIG. 3. Calibration curves for 15-*(S)*-HPETE, 18:0/20:4 PCOOH and 16:0/18:2 PCOOH. The separation and detection of hydroperoxides are described in the legend to Figure 2. Relative sensitivities of hydroperoxide detection relative to 15-*(S)*-HPETE (1.0) were for 18:0/20:4 PCOOH (1.0) and for 16:0/18:2 PCOOH (3.5). HPETE, hydroperoxyeicosatetraenoic acid; PCOOH, phosphatidylcholine hydroperoxides.

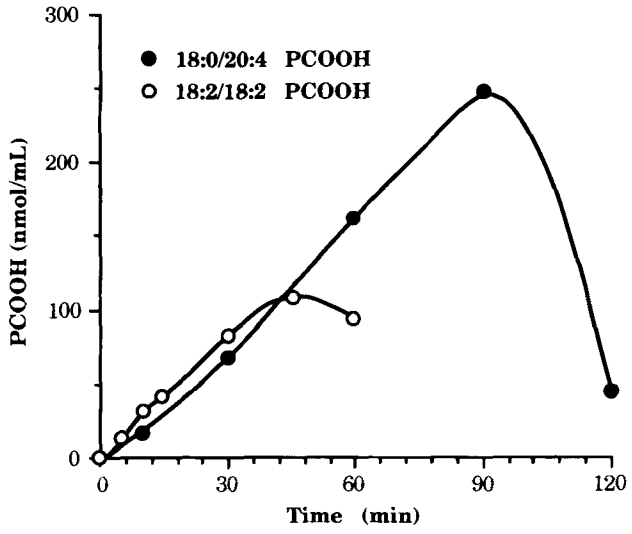

FIG. 4. Effect of incubation time with lipoxygenase on PCOOH production from 340 nmol of 18:2/18:2 PC (○) and 18:0/20:4 PC (●) per mL of incubation mixture. Abbreviations as in Figure 1.

Increasing concentrations (10–1000 nmol/mL of incubation mixture) of molecular species of standard or soybean PC and PE (18:2/18:2, 18:3/18:2, 18:0/20:4 and 16:0/18:2) were used to study the effect of phospholipid concentration on the rate of hydroperoxide production. Figure 5 shows the strong correlation between the amount of phospholipid present at T0 before peroxidation and that of the hydroperoxides detected after peroxidation. For a given concentration of 18:2/18:2 PC at T0, the concentration of hydroperoxides (Fig. 5A) was approximately twice that obtained with 16:0/18:2 PC (Fig. 5B), reflecting the importance of fatty acid unsaturation on hydroperoxide production.

The quantity of each molecular species transformed during 45 min of incubation was calculated as the difference between the concentration at T0 (start) and after 45 min of peroxidation (T45). This amount (T0–T45) was closely correlated to the concentration of the four molecular species at T0. Figure 6 shows the results obtained for 16:0/18:2 PC. Furthermore, the amount of phospholipid peroxidized was proportional to the number of double bonds in the constituent unsaturated fatty acids. For example, with 100 nmol of phospholipid at T0, 16.8 nmol of 16:0/18:2 PC was transformed, compared to 33.1 nmol of 18:2/18:2 PC (Table 1). We also assessed whether the amount of phospholipid transformed corresponded to the hydroperoxide concentrations determined by means of CL. Figure 7 shows, as an example, 16:0/18:2 PC, which was almost totally transformed into its hydroperoxide. Similar results were observed with 18:0/20:4 PC and 18:2/18:2 PC. However, this was only the case when the phospholipid concentrations at T0 were 50 nmol per mL or higher. At lower concentrations, the quantity of hydroperoxide produced was no longer related to the amount of molecular species transformed. Figure 7 shows that with 10–30 nmol/mL of phospholipid, similar amounts of hydroperoxide were detected. The procedure may not be suitable for

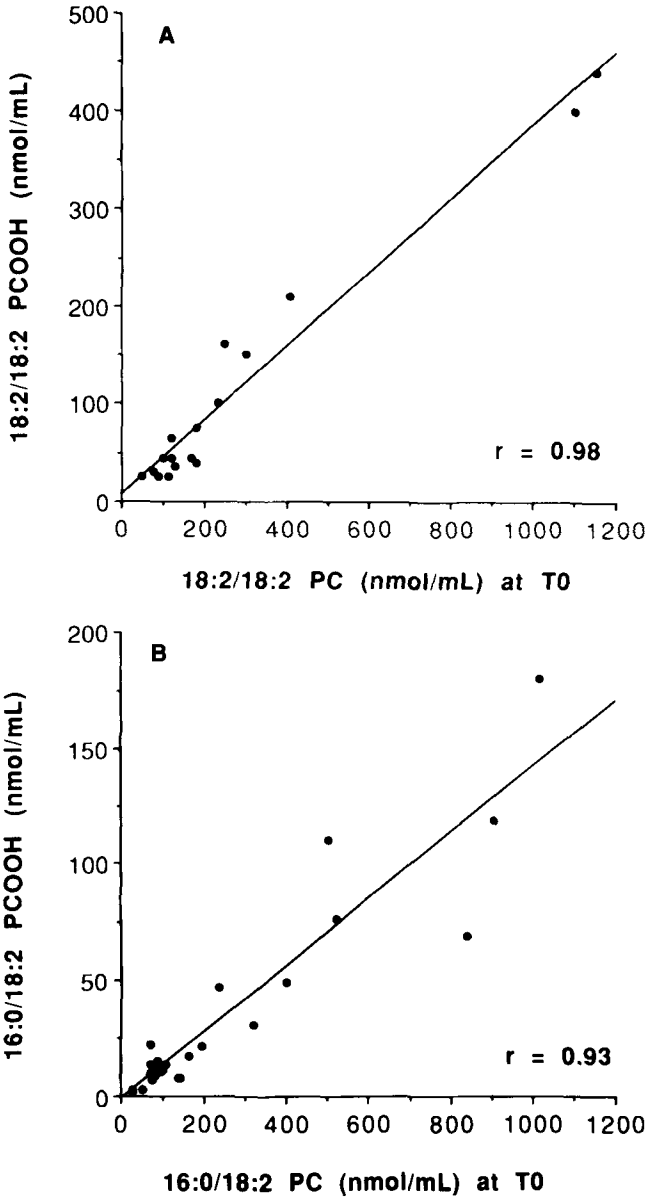

FIG. 5. Relationship between the concentration of 18:2/18:2 PC (A) and 16:0/18:2 (B) PC before peroxidation (T0) and the concentration of their corresponding PCOOH detected by chemiluminescence. Abbreviations as in Figure 1.

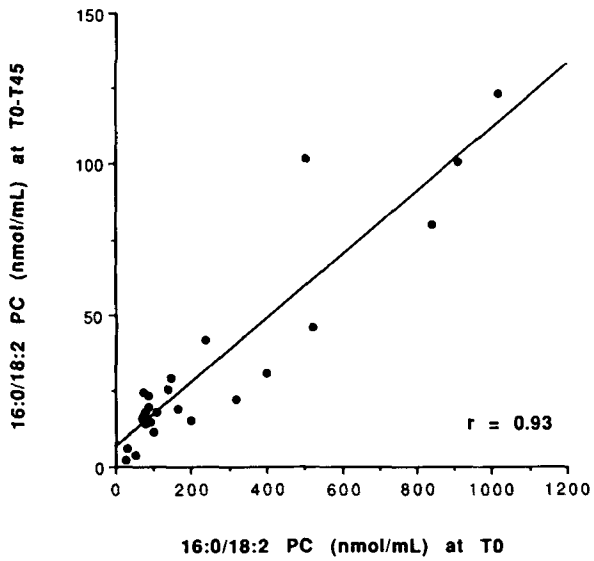

FIG. 6. Relationship between the concentration of 16:0/18:2 PC before (T0) and after peroxidation (T0–T45). Abbreviation as in Figure 1.

TABLE 1

Relationship Between the Amount of Phosphatidylcholine Molecular Species Transformed After Peroxidation and the Number of Double Bonds

Phospholipid molecular species (*sn*-1/*sn*-2)	Molecular species transformed[a] (nmol/mL)
18:3/18:2	42.3
18:2/18:2	33.1
18:0/20:4	30.5
16:0/18:2	16.8

[a]The concentrations of molecular species transformed were calculated from the regression line established for each molecular species, as shown in Figure 6, and expressed per 100 nmol of phosphatidylcholine per mL of incubation mixture.

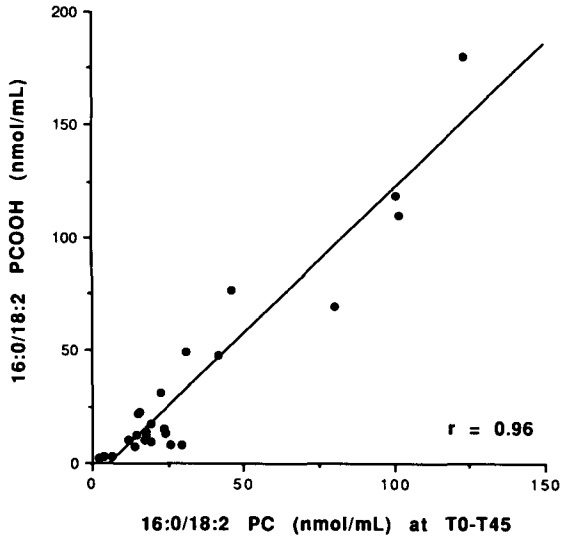

FIG. 7. Relationship between the concentration of transformed 16:0/18:2 PC after peroxidation (T0-T45) and the concentration of the corresponding PCOOH detected by means of chemiluminescence. Abbreviations as in Figures 1 and 2.

very low concentrations, possibly because the incubation time in the presence of lipoxygenase becomes too long, leading to degradation of the hydroperoxides produced.

In conclusion, the HPLC method described combines the separation of the main molecular species of phospholipids (PE, PC) and their corresponding hydroperoxides. The simultaneous determination of these products by spectrophotometry and CL allows quantification of both. The method should prove suitable for the analysis of molecular species in biological materials, such as in erythrocyte ghosts.

ACKNOWLEDGMENT

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Enantiomeric Resolution of Diacylglycerol Derivatives by High-Performance Liquid Chromatography on a Chiral Stationary Phase at Low Temperatures

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Separation of 1,2-diacylglycerol (DG) enantiomers as their 3,5-dinitrophenylurethane derivatives by high-performance liquid chromatography on a chiral column (Sumichiral OA-4100) was significantly improved at low temperature, *e.g.*, at −30°C. A linear relationship between logarithmic retention volumes and the number of carbons and olefinic bonds of the DG enantiomers was obtained. The effects of temperature on the separation are discussed.
Lipids 28, 251–253 (1993).

We have recently been able to separate various 1,2-diacylglycerol (1,2-DG) enantiomers by high-performance liquid chromatography (HPLC) on chiral stationary phases (CSPs) as their 3,5-dinitrophenylurethane (3,5-DNPU) derivatives (1–3). These separations have proven very useful, but some problems remain. For example, improved enantioselectivity would often be desirable for the analysis of natural lipid mixtures.

In the present study, the effect of temperature on the enantiomer separation of 1,2-*rac*-DG was investigated with the goal to improve resolution. The CSP used in the present study also resolves DGs according to carbon number and the number of double bonds of the acyl groups. The effects of temperature are described.

MATERIALS AND METHODS

Samples. Enantiomers of 1,2- and 2,3-*sn*-DG were synthesized by the method of Howe and Malkin (4). DG racemates were obtained by interesterification of fatty acid methyl esters with glycerol in dimethylformamide. Separation of 1,2-*rac*-DG was carried out by thin-layer chromatography on silica gel G impregnated with boric acid, using hexane/diethyl ether (60:40, vol/vol) and chloroform/acetone (96:4, vol/vol) as developing solvents. Conversion of 1,2-*rac*-DG to the 3,5-DNPU derivatives was accomplished as described previously (5,6).

HPLC. HPLC separations were carried out using a Hitachi L-6200 instrument (Hitachi Ltd., Tokyo, Japan) equipped with a Shimadzu SPD-6A UV detector (Kyoto, Japan) and a chiral column (stainless-steel, 50 cm × 4 mm i.d.) packed with 5-μm particles of *N-(R)*-1-(α-naphthyl)-ethylaminocarbonyl-*(S)*-valine chemically bonded to γ-aminopropyl silanized silica (Sumichiral OA-4100, Sumitomo Chemical Co., Osaka, Japan). Analyses were done isocratically using a mixture of hexane/dichloroethane/ethanol as mobile phase at a constant flow rate. A Hitachi 638-0805 recycling valve (Hitachi Ltd.) was used (3). The column was put in a cylindrical tube (45 cm in height, 9 cm in diameter) along with an immersion cooler (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The temperature was maintained within about ±1.0°C. The solvent was not precooled before the injection system or before the column.

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Abbreviations: CSP, chiral stationary phase; DG, diacylglycerol; 3,5-DNPU, 3,5-dinitrophenylurethane; HPLC, high-performance liquid chromatography; Vr, corrected retention volume; α, separation factor; Rs, peak resoltuion.

RESULTS AND DISCUSSION

Figure 1 shows the separation of a mixture of saturated 1,2-*rac*-DG homologues as 3,5-DNPU derivatives on Sumichiral OA-4100 at various temperatures. The homologue series was prepared by interesterification of a mixture of

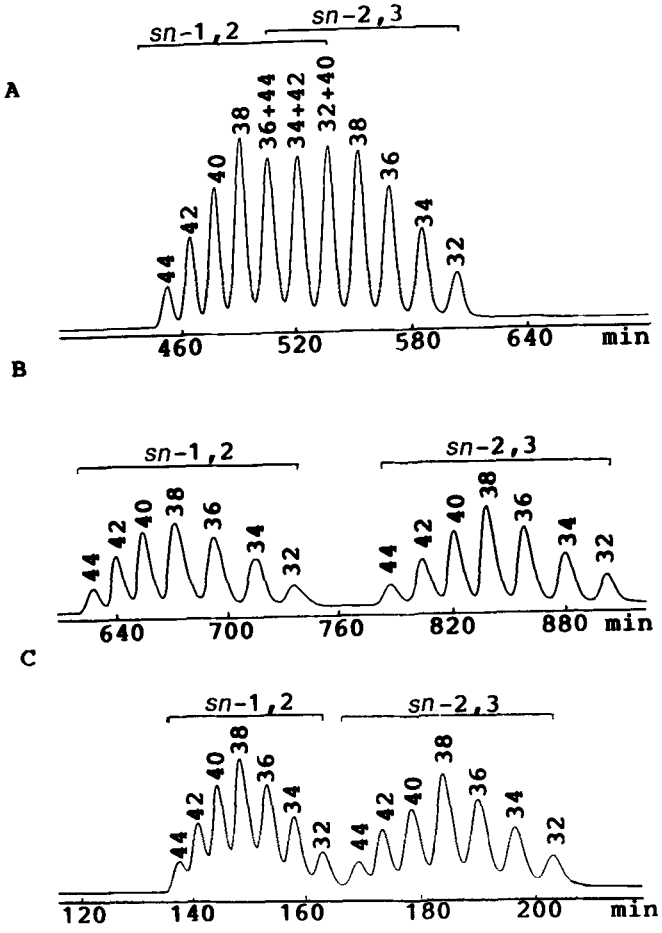

FIG. 1. Separation of saturated diacylglycerol (DG) enantiomers as 3,5-dinitrophenylurethane derivatives on an OA-4100 chiral column. A, temperature, 19.5°C; mobile phase, hexane/dichloroethane/ethanol (170:10:1, by vol); flow rate, 0.25 mL/min. B, temperature, −23.0°C; mobile phase, hexane/dichloroethane/ethanol (170:10:1, by vol); flow rate, 0.25 mL/min. C, temperature, −20.0°C; mobile phase, hexane/dichloroethane/ethanol (150:20:1, by vol); flow rate, 0.5 mL/min. Peaks were monitored at 254 nm after the first recycling. Above each peak the acyl carbon number of the DG is given.

four fatty acid methyl esters, namely 16:0, 18:0, 20:0 and 22:0, together with glycerol. The product therefore consisted of ten DG molecular species containing one or two of these four fatty acids. The mixture could be separated by HPLC on this CSP into seven DG homologues differing by two carbons in the acyl groups of each enantiomer because the CSP also provides for achiral separation according to carbon number and number of double bonds (3). Complete enantiomer separation of the seven DG homologues was obtained at −23°C (Fig. 1B), whereas the racemates were not separated on the same column at the higher temperature (19.5°C) (Fig. 1A). The separation factors (α) and peak resolutions (Rs) between the 1,2- and 2,3-enantiomers increased from 1.13–1.14 to 1.24–1.28, and from 4.33–5.07 to 9.64–11.13, respectively. For thermodynamic reasons (7), elution of the enantiomers was retarded by operating at the lower temperatures. We previously described (3) that the separation of enantiomeric 1,2-*rac*-DG homologues prepared from a mixture of those saturated fatty acids (16:0, 18:0, 20:0) required very long elution times (760 min) under the same chromatographic conditions as in Figure 1A.

In the present study, HPLC conditions were modified to accomplish a more rapid elution. This was done by selection of a more polar mobile phase and of a constant flow rate of 0.5 mL/min (Fig. 1C). The values of α and Rs of the enantiomers were 1.28–1.29 and 2.06–2.36, respectively.

Figure 2 shows the enantiomer separation by HPLC on the CSP as function of temperature of the 1,2-*rac*-DG prepared from 18:0 and 18:1 acids by transesterification with glycerol. Again, the better enantiomer resolution was obtained at the lower temperature (−10.5°C). The values of α and Rs for the enantiomers increased from 1.12 to 1.24, and from 3.41–3.44 to 6.05–6.28, respectively.

Very low HPLC column temperatures, *e.g.* −30°C as used in the separation shown in Figure 1B, often caused abnormal and sudden pressure increases, resulting in irregular chromatographic profiles and in peak deformations. These irregularities may in part be due to crystallization of the solutes and phase separation in the mobile phase consisting of a mixture of hexane, dichloroethane and ethanol. When these extra low temperatures were avoided, the retention times were reproducible and recoveries were quantitative.

Figure 3 shows the plots of the natural logarithm of retention volumes *vs.* acyl carbon numbers for the saturated DG. The two parallel lines are represented by the equations:

$$\ln \text{Vr}\ (sn\text{-}1,2) = h_c N + I \quad [1]$$

$$\ln \text{Vr}\ (sn\text{-}2,3) = h_c N + I + h_e \quad [2]$$

where corrected retention volume (Vr) (*sn*-1,2) and Vr (*sn*-2,3) are the retention volumes of 1,2- and 2,3-*sn*-DG enantiomers with the acyl carbon number N, respectively. The values of Vr were corrected by subtracting the column void volume; h_c (carbon number separation factor) and h_e (enantiomer separation factor) are the slopes of the lines and the distances between the two lines along the vertical axis, respectively. The value of I is a constant for the initial condition of Equation 1. This linear relationship between the logarithm of Vr and carbon numbers had also been found in the HPLC on CSP of a monoacylglycerol homologue mixture (8). The h_e value calculated from the linear relationship increased from 0.131 (24°C) to 0.230 (−12°C) by lowering the temperature. On the other hand, the value for h_c at 24°C and −12°C was 0.016. The results indicate that the separation of enantiomers is improved at lower temperature, while the resolution of DG molecular species according to carbon number is independent of temperature.

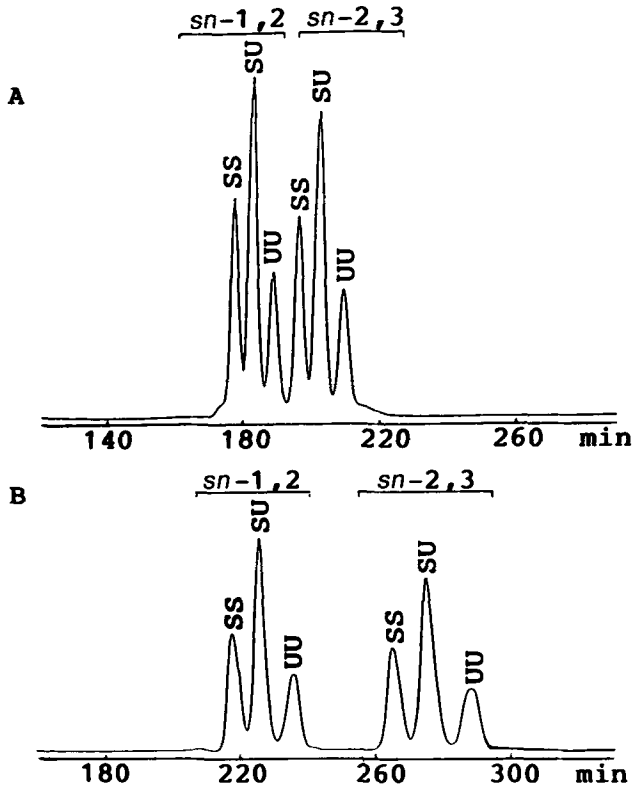

FIG. 2. Separation of diacylglycerol (DG) enantiomers prepared from stearic and oleic acids as 3,5-dinitrophenylurethane derivatives on an OA-4100 chiral column at different temperatures. A, 27.0°C. B, −10.5°C. Mobile phase, hexane/dichloroethane/ethanol (170:10:1, by vol); flow rate, 0.25 mL/min. SS, di-18:0-DG. SU, 18:0/18:1-DG. UU, di-18:1-DG. Peaks were monitored at 254 nm.

Figure 4 shows the plots of ln Vr *vs.* number of double bonds for DG prepared from 18:0 and 18:2 fatty acid mixtures. The linear relationships obey the equations:

$$h_d = \ln \text{Vr}\ (\text{SU-}sn\text{-}1,2) - \ln \text{Vr}\ (\text{SS-}sn\text{-}1,2)$$
$$= \ln \text{Vr}\ (\text{UU-}sn\text{-}1,2) - \ln \text{Vr}\ (\text{SU-}sn\text{-}1,2) \quad [3]$$

$$h_e = \ln \text{Vr}\ (sn\text{-}2,3) - \ln \text{Vr}\ (sn\text{-}1,2) \quad [4]$$

where S and U indicate saturated and unsaturated acyl groups, respectively; *e.g.*, SU stands for 18:0/18:2-DG. The h_e value is calculated from the distance between the two enantiomer lines, and the h_d value (double bond separation factor) is represented by the slope of the lines. Equation 3 was also applied to the *sn*-2,3-enantiomers, and the same h_d value can be expected for both antipodes as the lines for the two enantiomers are parallel. Higher values for h_e were obtained at lower temperature. The h_e value

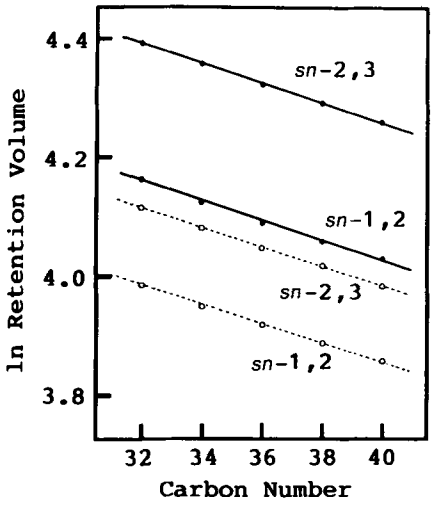

FIG. 3. Plots of ln retention volume *vs.* acyl carbon number for saturated diacylglycerol enantiomers as 3,5-dinitrophenylurethane derivatives on an OA-4100 chiral column at different temperatures. ●, −12.0°C. ○, 24.0°C. Mobile phase, hexane/dichloroethane/ethanol (170:10:1, by vol); flow rate, 0.25 mL/min.

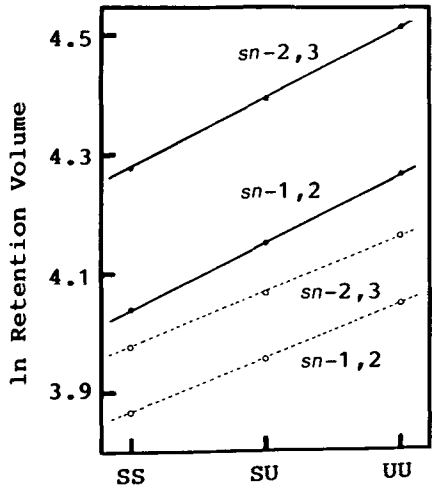

FIG. 4. Plots of retention volume *vs.* double bond number for diacylglycerol enantiomers prepared from stearic and linoleic acids as 3,5-dinitrophenylurethane derivatives on an OA-4100 chiral column at different temperatures. ●, −21.5°C. ○, 26.0°C. Mobile phase, hexane/dichloroethane/ethanol (170:10:1, by vol); flow rate, 0.25 mL/min.

of 0.245 obtained at −21.5°C is clearly much higher than that of 0.112 obtained at 26.0°C. In contrast, the h_d values increased from 0.092 (26°C) to 0.116 (−21.5°C) at lower temperature, that is, the slopes of the lines increased with decreasing temperature (Fig. 4). This trend was also noted for the other separations according to the number of double bonds, *e.g.*, for DG prepared from 18:0 and 18:3 acids. We found that the effect of temperature on the separation according to the number of double bonds increased with increase in the difference in number of olefinic bonds.

In present study we investigated the effects of temperature on the separations of DG according to enantiomeric configuration, carbon number and number of double bonds. We found that enantiomer separations were significantly improved by operating at low temperatures which give quantitatively correct and reproducible resolutions. Separation of DG molecular species according to carbon number was independent of temperature, whereas separation according to double bond was dependent on temperature.

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On-Line Straight-Phase Liquid Chromatography/Plasmaspray Tandem Mass Spectrometry of Glycerolipids

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Class separation of common glycerolipids on a diol-phase liquid chromatography column with a gradient of the mixed solvents hexane/isopropanol/acetic acid/triethylamine is compatible with on-line plasmaspray ionization mass spectrometry. The positive ion mass spectra exhibit prominent diacylglycerol and monoacylglycerol derived fragments, which can be utilized for quantification. In the selected-ion monitoring mode, the detection limit for phospholipids is in the low nanogram range. The abundance of the diacylglycerol and monoacylglycerol fragments reflects the relative fatty acid composition of the phospholipid classes. Collision-induced decomposition of diacylglycerol fragments with argon as collision gas unambiguously reveals the chain length and degree of unsaturation of the esterified fatty acids in the native lipid. *Lipids 28,* 255–259 (1993).

Modern mass spectrometry (MS) with soft ionization techniques including true thermospray (TSP), "filament on" TSP, fast atom bombardment (FAB) and, most recently, discharge-assisted TSP (plasmaspray, PSP) have opened new possibilities for the characterization of glycerophospholipids with high sensitivity. The ionization techniques now available have permitted detailed investigations which were recently reviewed (1).

Among the soft ionization techniques used for phospholipids, FAB has become especially popular as it can usually provide information on molecular weight and partial fatty acid composition (2–5). Most FAB investigations, however, have been concerned with phospholipid classes isolated by thin-layer chromatography, for example, and not with on-line high-performance liquid chromatography/mass spectrometry (HPLC/MS). Although dynamic FAB is rapidly gaining widespread use and can be utilized in combination with HPLC, the chromatographic procedure is still inconvenient. For example, low solvent flows and ionization matrices, such as glycerol, must be used, calling for splitting of standard HPLC effluents or the use of microbore HPLC columns.

However, by using "filament on" TSP and conventional wide-bore HPLC/MS with ammonium acetate as electrolyte and a high percentage of hydrophobic solvents, Kim *et al.* (6–8) obtained detailed structural information on phospholipids, including the diacylglycerol/monoacylglycerol-derived fragments and acylium ions.

We recently showed (10) that the PSP ionization technique can provide significant advantages over "filament on" TSP as used by Kim and Salem (7) in regard to sensitivity and the range of HPLC mobile phases available. Our approach was to apply an HPLC procedure which would permit a class separation only. We examined two HPLC systems (11,12) of which we chose a cation exchange column and an acetonitrile/methanol/water mixture in an isocratic mode.

Quite recently, Herslöf *et al.* (13) have significantly improved class separations of a variety of glycerolipids, including the common intact glycerophospholipids, on a diol column with a gradient system involving hexane, isopropanol, acetic acid, triethylamine and water as mobile phase. The superiority of this separation procedure over that involving a cation-exchange column (10) led us to investigate its merits in combination with PSP ionization.

As previously pointed out (10), the TSP and PSP ionization methods frequently do not give the fatty acid composition of the intact glycerolipid unambiguously, based on the mass fragments formed. The number of combinations of fatty acids possible for a specific mass number of a diacylglycerol-derived fragment increases substantially with multiple unsaturation. The advantages of using on-line HPLC/MS/MS for the analysis of biological samples are presently being explored in our laboratories. To increase the structural information that can be obtained, we decided to study precursor/product relationships of glycerolipids upon collision of diacylglycerol-derived ions with certain reagent gases. The present paper describes experiments on the use of HPLC/MS/MS for structure determinations of intact glycerolipids.

MATERIALS AND METHODS

Chemicals and solvents. Triolein, 1,2-dilauroyl-3-stearoyl-*sn*-glycerol (12:0/12:0/18:0), 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol (16:0Et/14:0/14:0), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate [16:0/16:0-phosphatidic acid (PA)], 1,2-distearoyl-*sn*-glycero-3-phosphocholine [18:0/18:0-phosphatidylcholine (PC)], 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine [16:0/18:2-phosphatidylethanolamine (PE)], 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1/18:1-PE) and phosphatidylinositol [soybean phosphatidylinositol (PI)] were used as reference compounds.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphate, 1,2-distearoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine were from Serdary Ltd. (London, Ontario, Canada), triolein and PI were purchased from Larodan (Malmö, Sweden). 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine were purchased from Sigma (St. Louis, MO). 1,2-Dilauroyl-3-stearoyl-*sn*-glycerol and 1-*O*-hexadecyl-2,3-dimyristoyl-*sn*-glycerol were prepared in the laboratory (14). All reference materials were stored under N_2 at −20°C until used.

The solvents used were from FSA Lab Supplier (Loughborough, England), or from May and Baker Ltd. (Dagenham, England). They were of HPLC grade and were used without further purification.

Bacterial samples. Pseudomonas fluorescens cells were grown, harvested and extracted for total lipids by Bligh

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Abbreviations: CID, collision induced decomposition; FAB, fast atom bombardment; HPLC/MS, high-performance liquid chromatography/mass spectrometry; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PSP, plasmaspray; TIC, total ion current; TSP, thermospray.

and Dyer (9) extraction as described previously (10). Freeze-dried cells (600 mg) yielded phospholipids which were dissolved in 1 mL of mobile phase (A) (see below).

Liquid chromatography. Class separations were performed by using a column packed with diol-modified silica (E. Merck, Darmstadt, Germany; Li-Chrosphere 100 Diol, 5 μm, 25 cm × 4 mm i.d.). The mobile phase was a modification of the method developed by Herslöf *et al.* (13) and consisted of (A) hexane/isopropanol/acetic acid/triethylamine (75:25:7.5:0.05, by vol) and (B) hexane/isopropanol/acetic acid/triethylamine (21:78:1.5:0.05, by vol) in a linear gradient from 100% of (A) to 100% of (B) during 25 min at a flow rate of 0.8 mL/min (13).

A Waters (Milford, MA) model 600-MS HPLC pump equipped with a variable-volume Waters model U6K injector was used. Samples injected were typically 10–20 μL from solutions containing 1 μg/μL of phospholipids.

MS. The effluent from the column was introduced into a Trio 3 (VG Masslab, Altrincham, United Kingdom) tandem quadrupole mass spectrometer through the standard liquid chromatography/MS thermospray/plasmaspray probe. The vaporizer capillary was at a temperature of 240–260°C, the ion source was at 280°C, the discharge current 500–700 μA and the repeller voltage typically between 95–110 V depending on source conditions. Collision-induced decomposition (CID) was achieved by using helium, argon or xenon at pressures between 1×10^{-4} to 5×10^{-2} mbar (measured in the collision cell) and at energies between 0-20 eV. A standard VG 11/253 data system configuration was employed for mass spectral data acquisition.

A cold trap (liquid nitrogen) was installed between the source diffusion pump and the backing rotary pump to prevent contamination of the pump oil by the hydrophobic HPLC solvents used.

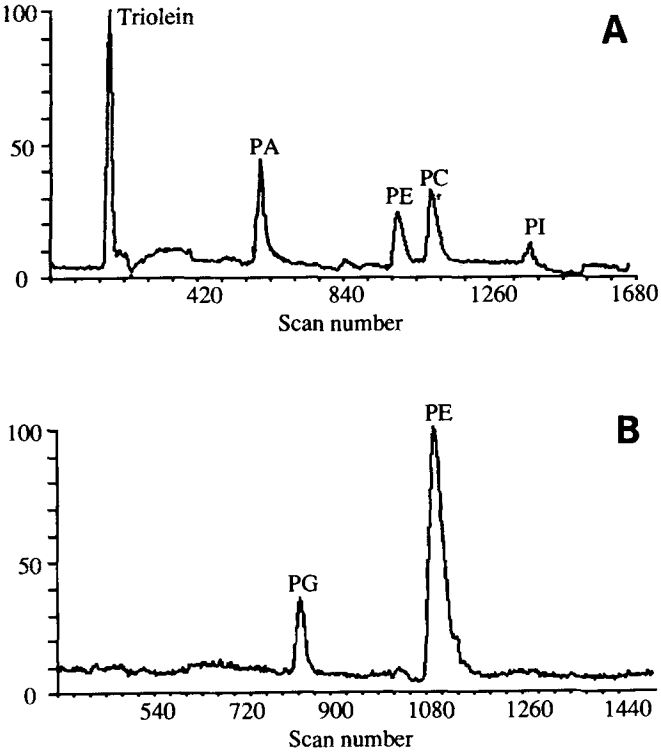

FIG. 1. A. Positive ion plasmaspray mass chromatogram of a mixture of glycerolipids consisting of: triolein, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (PA), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (PC) and phosphatidylinositol (PI from soybean). B. Positive ion plasmaspray mass chromatogram of a total Bligh and Dyer (9) lipid extract from *Pseudomonas fluorescens* cells showing two peaks containing phosphatidylglycerol (PG) and PE species.

RESULTS AND DISCUSSION

Separation of glycerolipid classes. The positive ion mass chromatogram in PSP [total ion current (TIC) scanning over the range of *m/z* = 120–600] using the diol column and gradient mobile phase system as described above is shown in Figure 1A. The chromatographic peaks represent 76, 24, 25, 25 and 10 μg for triolein, PA, PE, PC and PI, respectively. Good chromatographic peak shape is obtained for all glycerolipid classes studied, including PI which is a natural complex species mixture (see below) isolated from soybeans. Moreover, the gradual change of solvent composition during the chromatographic run does not require modification of the ion source PSP parameters.

A phospholipid extract from bacterial cells *(P. fluorescens)* was also studied by PSP under the same chromatographic conditions (Fig. 1B). Only two glycerophospholipid classes are distinguishable, phosphatidylglycerol (PG) and PE, in agreement with the previously-reported phospholipid composition (15). As both mass chromatograms indicated excellent class separation characteristics with negligible or no apparent molecular species separation (that is PI in Fig. 1A, PG and PE in Fig. 1B), the ions forming the PE peak (Fig. 1B) from *P. fluorescens* (scan numbers 1100–1150) were studied in some detail. The abundances of the diacylglycerol-derived fragment cluster (*m/z* = 540–610) in all spectra within the PE peak were plotted three-dimensionally as a function of the scan numbers (Fig. 2). The diacylglycerol-related portion of the PSP mass spectrum averaged over the entire peak is shown on top. The results indicate a slightly shorter retention time of phospholipids with longer fatty acid chains (*i.e.*, *m/z* = 604 corresponding to 36 fatty acid carbon atoms) compared to phospholipids with shorter fatty acids (*m/z* = 550 corresponding to 32 fatty acid carbon atoms).

Relative fatty acid composition. The fatty acid composition of a PI from soybean was calculated by measuring the mean ion intensities of the monoacylglycerol and diacylglycerol related fragments from a series of full-scan spectra of variable amounts (5, 12.5, 25 and 32 μg) of phospholipid. In Figure 3, the amount of fatty acids present represented as wt% of the total fatty acids, has been reproduced as calculated either from the monoacylglycerol related ions or from the diacylglycerol related ions. For comparison, results from conventional gas chromatographic fatty acid analysis after transesterification (Larodan, Malmö, Sweden) have been incorporated in the figure. Apart from minor components (*i.e.*, 18:3) which fall below the detection limit in full-scan, there is a good agreement between MS measurements on intact phospholipids and gas chromatography measurements of total fatty acid methyl esters.

PSP spectra of triacylglycerol and 1-O-alkyl-2,3-diacyl-sn-glycerols. We have also studied the MS fragmentation of some nonphosphatidic glycerolipids using PSP

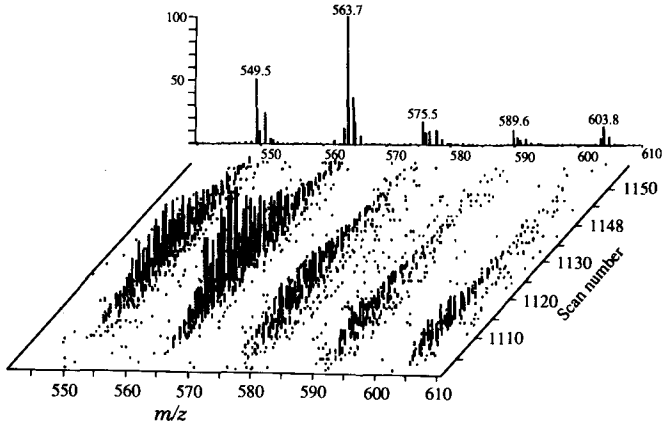

FIG. 2. Three-dimensional presentation of the diacylglycerol-derived fragments (m/z = 540–610) extracted from the phosphatidylethanolamine (PE) peak in Figure 1B. The upper spectrum shows the average intensities of the peak.

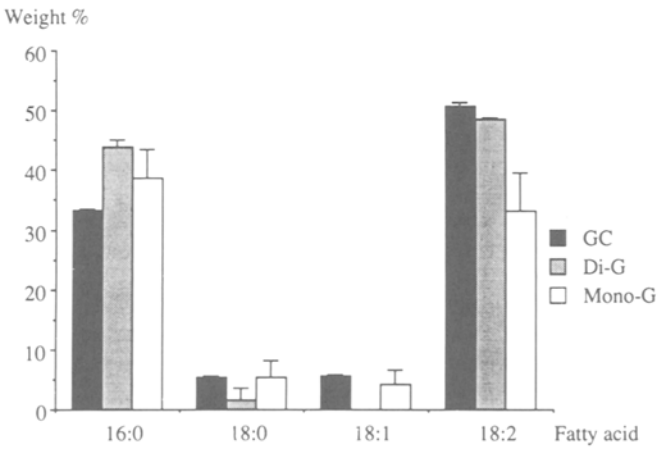

FIG. 3. Fatty acid composition of phosphatidylinositol from soybean determined by gas chromatography (GC) and by plasmaspray mass spectrometry calculated from the total counts of the intensities of the diacylglycerol-derived (Di-G) and the monoacylglycerol-derived (Mono-G) fragments.

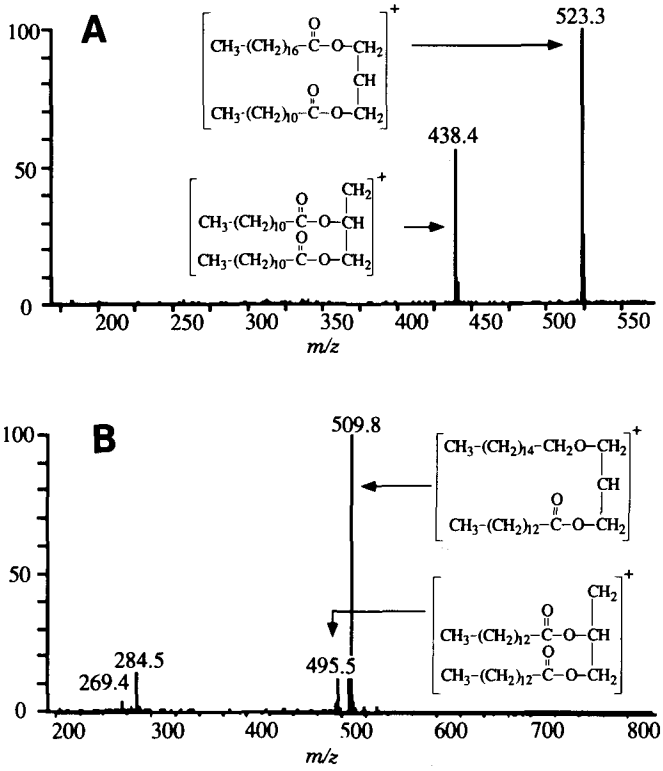

FIG. 4. Positive ion plasmaspray spectrum of A, 1,2-dilauroyl-3-stearoyl-*sn*-glycerol, and B, 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol.

ionization. Figure 4A shows the positive ion PSP spectrum of 10 μg of 1,2-dilauroyl-3-stearoyl-*sn*-glycerol. As for the phosphatides, no $(M + H)^{+1}$ ions are seen in PSP. The spectrum shows only the two possible diacylglycerol related fragments, formed by loss of a fatty acid from either position of the protonated molecule.

In Figure 4B, a spectrum of 1-*O*-hexadecyl-2-3-dimyristoyl-*sn*-glycerol has been reproduced. Similar cleavage mechanisms as operate for the triacylglycerol are seen. Notably, cleavage with loss of the carboxylic acid (α- and β-carbon atom) from the protonated molecule (m/z = 509) is a more prominent process than that involving formal loss of the alcohol group (m/z = 495).

Quantification. Previous preliminary studies on the quantification of phospholipids by using PSP and the cation HPLC column with an acetonitrile/methanol/water mixture indicate that straightforward procedures can be used to give linear dose-response curves. Such studies were repeated in some detail for several phospholipid classes by using the diol column and the hydrophobic solvent mixtures used in this work. Figure 5 shows dose response curves for PA, PC and PE (all dipalmitoyl substituted) measured by single ion monitoring of the m/z = 551 ion (diacylglycerol related fragments). The responses exhibit good linearity for all molecular species ($r^2 > 0.99$), and an intercept at the origin indicates negligible adsorption and/or breakdown in the chromatographic and interface system. At a signal-to-noise ratio of 3:1, the detection limit is approximately 3 ng. The ionization tendencies for the phospholipid classes investigated are in the same order of magnitude. Sensitivity in selected ion monitoring (SIM) is comparable with that obtained in the previously used liquid chromatographic system, providing a detection limit in the low nanogram range (10).

In another experiment, we compared the sensitivity of two PC molcules with different chain-lengths (dipalmitoyl and dioctanoyl PC). We found that the relative sensitivity between the long and short chain lengths is dependent on the experimental conditions, *i.e.*, tuning of the ion source, the cleanness of the source and the capillary vaporizer, but reproducibility under set conditions was found to be satisfactory.

CID measurements. PSP ionization of a phospholipid with a defined fatty acid composition was studied. Figure 6A shows the PSP positive ion mass spectrum of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine. As expected, diacylglycerol related fragments are present at m/z = 575 (18:2 + 16:0). The spectrum further shows two monoacylglycerol related fragments at m/z = 337 (18:2) and m/z = 313 (16:0).

The mass spectrometer was arranged for MS/MS measurements in the precursor/product mode. A CID mass

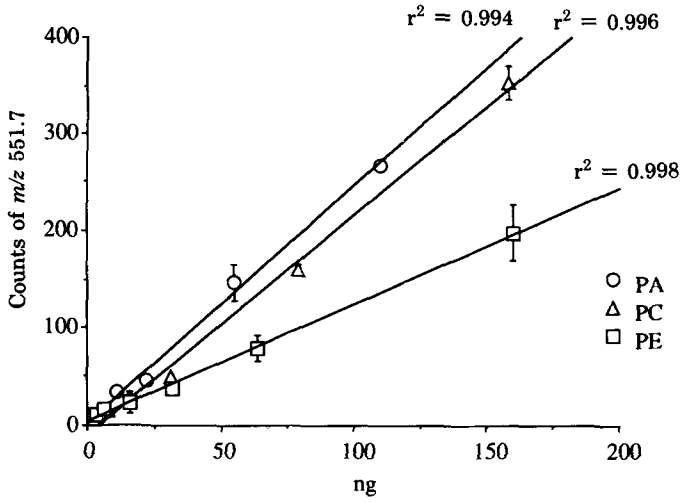

FIG. 5. Dose response curves of the diacylglycerol related fragments (*m/z* = 551) from 1,2-dipalmitoyl-*sn*-glycero-3-phosphate [16:0/16:0-phosphatidic acid (PA)], 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [16:0/16:0-phosphatidylcholine (PC)], and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [16:0/16:0-phosphatidylethanolamine (PE)] measured in single ion mode.

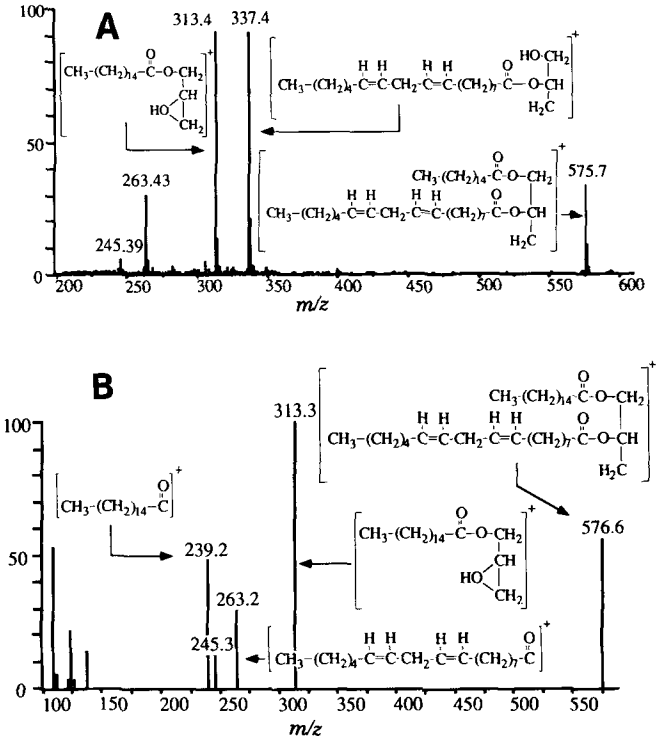

FIG. 6. A. Positive ion plasmaspray of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanolamine. B. Collision-induced decomposition of the diacylglycerol-derived fragment *m/z* = 575.5, showing the most abundant product ion, *m/z* = 313.3

spectrum of the diacylglycerol related fragments (*m/z* = 575) using argon as collision gas at a pressure of 10^{-3} torr, and a collision energy of 3 eV is shown in Figure 6B. The spectrum indicates in the high mass range the presence of undecomposed precursor ions at *m/z* = 575 and prominent monoacylglycerol related fragments at *m/z* = 313. Acylium ions at *m/z* = 263 and 239 of both fatty acids are observed in the spectrum corresponding to 18:2 and 16:0 fatty acids, respectively. The spectrum further indicates subsequent fragmentation of the fatty acid residues leading to a series of low mass fragments. The CID experiment thus unequivocally reveals fatty acid composition.

As an application, the fatty acid composition of PI from soybeans was examined. The three most abundant diacylglycerol related fragments as indicated from the full spectrum (Fig. 7) were analyzed by using precursor/product MS/MS as described above.

The three MS/MS product ion spectra of the precursor diacylglycerol related ions as shown in Figure 7 give information on the fatty acid species. The diacylglycerol related ions of *m/z* = 575.6 consist of an 18:2 fatty acid moiety (acylium ions of *m/z* = 263.3) and a 16:0 fatty acid (monoacylglycerol related ion of *m/z* = 313.3). Analogously, the H_2O adduct of the diacylglycerol related ions of *m/z* = 591.7 consists of an 18:3 acylium ion (*m/z* = 261.2) and a 16:0 acid (*m/z* = 313.3). The diacylglycerol-related ions of *m/z* = 603.3 have an 18:0 acid (*m/z* = 341) and a 18:2 acid (*m/z* = 263.3).

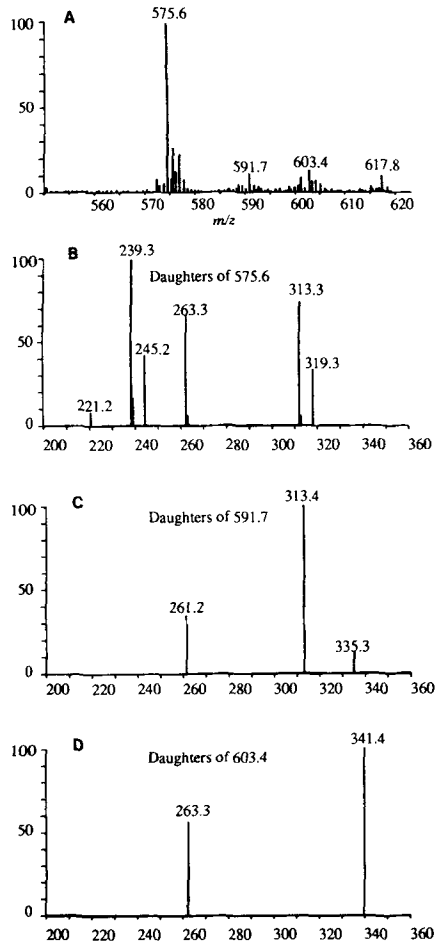

FIG. 7. A. Mass spectrum of phosphatidylinositol from soybean, and B, the collision-induced decomposition product ion spectra of the precursor ions of *m/z* = 575.6, C, of *m/z* = 591.7, and D, of *m/z* 603.4.

Changing the collision energy indicates that useful information was obtained only within a rather narrow ion energy range. For example, at 3 eV there is practically no decomposition of the precursor fragments whereas at 20 eV the CID spectrum only showed unspecific fragments of low mass. There are no major qualitative differences in the spectra when the collision gas Ar was replaced by He or Xe as long as the collision energy and pressure are adjusted accordingly for each. Argon was found to be the collision gas of choice, because adequate fragmentation was obtained at convenient gas pressures and collision energies.

CID after PSP ionization offers excellent possibilities to study glycerophospholipid molecules in detail. It is possible to obtain information on the fatty acid composition of individual species within phospholipid classes.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Natural Science Research Council. Valuable discussions with Dr. Mark McDowall at VG Biotech, Altrincham, United Kingdom, and the skillful technical assistance of Gunilla Westerdahl are gratefully acknowledged.

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A Thermospray Mass Spectrometric Assay for Fe-Induced 4-Hydroxynonenal in Tissues

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A method is presented for the determination of 4-hydroxynonenal (HNE) in tissue homogenates following *in vitro* lipid peroxidation induced by iron (Fe^{++}). HNE is measured as the pentafluorobenzyl oxime derivative using liquid chromatography thermospray mass spectrometry. *In vitro* metabolism of HNE *via* the glutathione/glutathione-*S*-transferase pathway was inhibited using iodoacetic and iodobenzoic acids. The assay has been used as an indicator of the peroxidizability of tissue samples from animals both adequate in and depleted of α-tocopherol. The concentrations of HNE produced in tissues taken from animals depleted of α-tocopherol were found to be up to 8 times higher than those taken from animals supplemented with α-tocopherol.
***Lipids* 28, 261-264 (1993).**

Lipid peroxidation has been implicated in causing a wide range of biological effects (1,2) including heart disease, aging, retinal degeneration and cancer. Many tests have been proposed as indicators of lipid peroxidation in biological systems. Techniques for measuring primary lipid peroxidation products such as lipid hydroperoxides have included high-performance liquid chromatography (HPLC) with electrochemical (3) or chemiluminescent (4) detection and gas chromatography/mass spectrometry (GC/MS) of the methyl, trimethylsilyl and pentafluorobenzyl (PFB) esters of the hydroxy derivatives of the lipid hydroperoxides (5). Assays have also been developed to measure secondary lipid peroxidation products such as thiobarbituric acid reactive substances (TBARS; Ref. 6), conjugated dienes (7), alkanes such as ethane and pentane (8), and aldehydes such as hexanal (9) and 4-hydroxynonenal (HNE; Refs. 10,11).

The latter aldehyde is formed through the oxidation of linoleic or arachidonic acid (12,13) and has received much attention in the last few years. It has been shown to be cytotoxic and can exhibit a wide range of potent biological effects (14-17). It is also highly reactive toward molecules containing sulfhydryl groups such as reduced glutathione (GSH), cysteine and proteins in general (18,19).

Several techniques have been proposed for measuring HNE in biological samples. These methods have recently been reviewed (20) and include HPLC of the fluorescent decahydroacridine derivatives of HNE (21) and GC/MS of the butyldimethylsilyl (22) or PFB oxime derivatives (10,11). However, as mentioned above, HNE is highly reactive toward a wide range of cellular components. One study by Ishikawa *et al.* (23) showed that the half life of HNE in perfused rat heart was less than 4 s as a result of the conjugation of HNE to GSH, catalyzed by glutathione-*S*-transferase (GSHT) [E.C. 2.5.1.18]. Therefore it is unclear what proportion of the HNE originally present in the tissues can be recovered and measured by any of these procedures, since any free HNE may have been rapidly metabolized or irreversibly bound to cellular macromolecules. This problem is compounded by the possibility that some HNE may be formed during the work-up procedures. An alternative approach would be to analyze HNE in tissue samples in a way similar to that of measuring TBARS following ascorbate- or Fe^{++}-induced *in vitro* lipid peroxidation (24). This paper describes a method in which tissue homogenates are first treated with inhibitors of GSHT and then incubated with Fe^{++} to induce the production of HNE. This aldehyde is then measured using liquid chromatography/thermospray mass spectrometry (LC/MS) following derivatization with PFB hydroxylamine hydrochloride. The method has been used to measure the *in vitro* production of HNE following Fe^{++}-initiated peroxidation of muscle homogenates from cattle and pigs either supplemented with or depleted of α-tocopherol.

MATERIALS AND METHODS

Materials. HNE was kindly provided by Professor H. Esterbauer, Department of Biochemistry, University of Graz, Austria. It was supplied as a 50-60 mmol/L stock solution in dichloromethane and was stored at −20°C until required. An aliquot of this solution (500 μL) was evaporated to dryness under nitrogen at room temperature. The residue was dissolved in water (5 mL) by shaking. This solution was used as a stock standard and was stable for several months at 4°C. The exact concentration of HNE in this solution was determined by diluting 100 μL with 9.9 mL water and recording the ultraviolet spectrum in a 1-cm cuvette from 200-300 nm. The concentration was determined using the molar extinction coefficient of 13750 at 223 nm. Dilute standards (10 μM) were freshly prepared from the stock standard in water as required.

Piperazinediethanesulfonic acid (PIPES), PFB hydroxylamine hydrochloride, iodoacetic acid (IAA) and *o*-iodobenzoic acid (IBA) were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, tetrahydrofuran, methanol and hexane were all HPLC-grade.

Tissue α-tocopherol concentrations. These were measured using HPLC with fluorescence detection, essentially as described by McMurray and Blanchflower (25).

Extraction of HNE from tissue and derivatization. Samples of tissue (0.5 g) were homogenized in ice-cold 25 mM PIPES buffer, pH 7.0 (9.5 mL), using a Silverson

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Abbreviations: Fe^{++}, iron(II) ion; GC/MS, gas chromatography/mass spectrometry; GSH, reduced glutathione; GSHT, glutathione-*S*-transferase; HNE, 4-hydroxynonenal; HPLC, high-performance liquid chromatography; IAA, iodoacetic acid; IBA *o*-iodobenzoic acid; LC/MS, liquid chromatography/thermospray mass spectrometry; PFB, pentafluorobenzyl; PIPES, piperazinediethanesulfonic acid; SIM, selected ion monitoring; TBARS, thiobarbituric acid reactive substances.

homogenizer (Silverson Machines, Chesham, United Kingdom) for about 1 min, followed by 10 strokes in a Dounce homogenizer (Thomas Scientific, Swedesboro, NJ). Samples were freshly prepared and kept on ice at all times.

Aliquots of homogenate (1.0 mL) were transferred to test tubes to each of which was added mixed inhibitor solution (100 μL) containing 10 mM IAA and 10 mM IBA, dissolved in water. Following incubation for 10 min at 37°C in a shaking water bath, iron(II) sulfate (100 μL of a 7.2 mM aqueous solution) was added, and the samples were incubated for a further 45 min. Methanol (2 mL) was added, and the tubes were centrifuged at 2000 × *g* for 10 min at 4°C. Aliquots of supernatant (2.0 mL) were transferred to clean tubes. Standards (50 μL of 10 μM HNE) were also set up at this point, and the volumes of the tubes were adjusted to 2.0 mL using methanol/water (2:1, vol/vol). Aliquots of PIPES buffer (1.0 mL of a 25 mM aqueous solution, pH 7.0) and PFB hydroxylamine hydrochloride (100 μL of a 50 mg/mL freshly prepared solution in methanol) were added to all tubes. After standing at room temperature for 30 min, the mixtures were extracted with hexane (2 × 1 mL) by shaking for 30 s and centrifuging at 2000 × *g* at 4°C for 10 min. The hexane extracts were combined and evaporated to dryness under nitrogen at 50°C. The residues were dissolved in HPLC mobile phase (100 μL).

Analysis of HNE using thermospray LC/MS. The HPLC system consisted of a Merck-Hitachi (BDH Ltd., Dagenham, United Kingdom) model L-6000 pump, a Rheodyne (Rheodyne Inc., Cotati, CA) model 7125 injector fitted with a 50 μL sample loop and a Lichrosorb (BDH Ltd.) RP18, endcapped, 125 × 4 mm reverse-phase cartridge with holder. The mobile phase, acetonitrile/tetrahydrofuran/water (75:5:20, by vol), was pumped at a rate of 1.0 mL/min. This system was directly coupled to a Vestec model 201A thermospray LC/MS (Vestec Corporation, Houston, TX) complete with a Technivent workstation. The instrument was operated in the negative ion mode, using filament-initiated ionization with an electron beam current of 250 μA. The electron multiplier voltage was 1600 V. The temperatures of the source block, tip heater and lens assembly were 280, 250 and 150°C, respectively. The vaporizer probe was operated at about 15°C below the take-off point. This was optimized for each probe used but was typically in the region of 170°C. The instrument was operated either in the full scan mode to collect spectra, or in the selected ion monitoring (SIM) mode for maximum sensitivity.

Aliquots (25 μL) of the derivatized standards and sample extracts were injected into the LC/MS system. A standard was injected after every four samples to correct for drift, if any. SIM data were collected for the negative ion at *m/z* 331. The concentrations of HNE in samples were calculated from peak areas by comparison with standards.

Data. Where appropriate, data are presented as means ± SE. Comparisons between the α-tocopherol and HNE concentrations in tissue were by Student's *t*-test.

RESULTS AND DISCUSSION

The thermospray LC/MS negative ion spectrum for the PFB derivative of HNE is shown in Figure 1, together with the molecular structure of this derivative. This spectrum is unusual for thermospray LC/MS, when the base

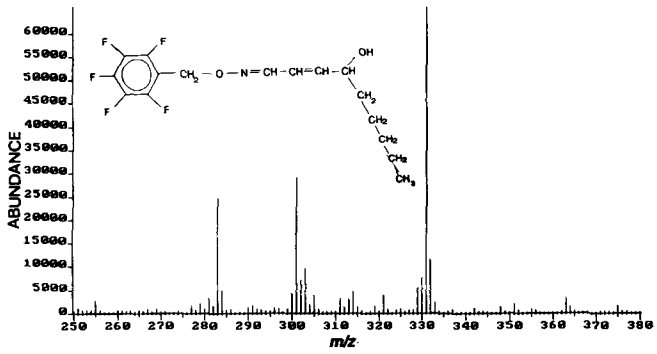

FIG. 1. Negative ion liquid chromatography/thermospray mass spectrometry spectrum and structure of the pentafluorobenzyl hydroxylamine derivative of 4-hydroxynonenal.

peak is normally the molecular ion (M), M + 1, or M − 1. In this case only a small molecular ion at *m/z* 351 is seen. The base peak fragment ion at *m/z* 331 probably results from the loss of hydrogen fluoride from the molecule and is the most sensitive ion to use for SIM. It is interesting to note that the best sensitivity is obtained without the addition of ammonium acetate to the HPLC mobile phase, a normal requirement of LC/MS.

Few authors have quoted recovery values for HNE from whole tissue homogenates. Norsten-Höög and Cronholm (22) reported a recovery of 84% from hepatocytes. They did not, however, quote a figure for liver homogenates, but gave a recovery value of 60% for their internal standard, cyclohexanone.

In the present study, initial attempts to measure analytical recoveries were disappointing. Therefore two approaches were attempted to improve the recovery of HNE from tissue homogenates, namely disruption of protein binding and inhibition of the GSH/GSHT conjugation pathway.

Hydroxylamine (22) or PFB hydroxylamine (11) have previously both been added to biological samples to recover HNE conjugated to protein, but the authors offered no evidence as to the efficacy of these procedures. We observed that HNE added to tissue homogenates disappears rapidly (80% in 5 min) and could not be recovered by these techniques (data not shown). It would therefore appear that protein conjugation is not the major problem causing poor recoveries, but that HNE is being rapidly metabolized *in vitro* by tissue homogenates.

Ishikawa *et al.* (23) have shown that HNE can react spontaneously with GSH, but that the rate of the reaction is increased 30-fold when catalyzed by GSHT. These workers estimated the half life of HNE in heart as less than 4 s, under the conditions used. Alin *et al.* (26) concluded that spontaneous reaction between GSH and HNE was negligible and that one of the major functions of GSHT was to protect the cell from the cytotoxic action of aldehydes such as HNE. However, Pabst *et al.* (27) showed that the GSHT-catalyzed conjugation of HNE to GSH could be inhibited by IAA and IBA. The effect of adding these substances to tissue homogenates carried through the described assay is shown in Figure 2. In the untreated homogenate, there was no increase in the HNE concentration for the first 30 min of incubation time, whereas in the inhibitor-treated homogenate there was a steady increase in HNE production. This showed that the

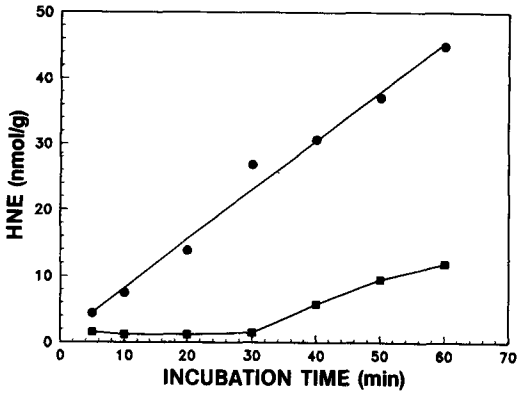

FIG. 2. Time course of the *in vitro* Fe^{++}-induced production of 4-hydroxynonenal by a tissue homogenate with (●) or without (■) incubation with iodoacetic and iodobenzoic acids as inhibitors of glutathione-*S*-transferase.

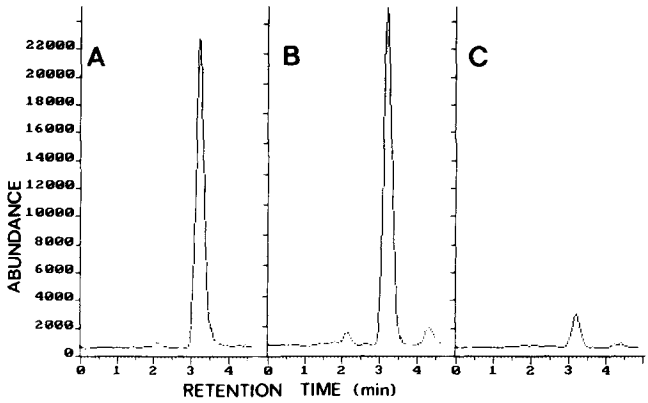

FIG. 3. Selected ion monitoring chromatograms of the negative ion at *m/z* 331 for the pentafluorobenzyl hydroxylamine derivative of 4-hydroxylnonenal from (A) a 10 μM standard solution, (B) a heart muscle homogenate from a α-tocopherol deficient pig and (C) a heart muscle homogenate from an α-tocopherol supplemented pig. The assays were performed as described in the text. 4-Hydroxylnonenal eluted from the column at a retention time of 3.2 min.

HNE produced in the untreated homogenate was being rapidly metabolized by the GSH/GSHT pathway; a rise only became apparent after 30 min, possibly as a result of GSH depletion. The inhibitors IAA and IBA therefore blocked this reaction in the treated homogenate which showed a 4-fold increase in HNE concentration after a 45-min incubation period, when compared with untreated homogenate.

In the described assay, recovery values were determined by spiking muscle homogenates with HNE, following treatment with IAA and IBA, incubation with Fe^{++} and then proceeding as described in the assay. The recovery values averaged 65% (Table 1), confirming that IAA and IBA had effectively blocked the reaction with GSH/GSHT. The losses of HNE (35%) may have been due to binding to other macromolecules, but this could not be confirmed.

Single ion chromatograms of the ion at *m/z* 331 are shown in Figure 3 for a standard and for heart muscle homogenates from α-tocopherol deficient and α-tocopherol supplemented pigs. The HNE derivative eluted from the column with a retention time of 3.2 min and was apparently free from interference by other compound eluting closely. The standard is equivalent to 16.1 nmol HNE/g sample. The concentrations of HNE induced in the muscle samples from the α-tocopherol deficient and α-tocopherol supplemented pigs were 17.8 and 1.9 nmol/g, respectively.

TABLE 1

Recovery of HNE from Muscle Homogenate[a]

HNE added (nmol)	HNE recovered (nmol)	Recovery (%)
0.00	0.10	—
0.32	0.32	66
0.64	0.51	64
1.28	0.93	65

[a]Aliquots (1.0 mL) of muscle homogenate, with added 4-hydroxynonenal (HNE) at the levels indicated, were carried through the described assay. Results are the mean of duplicate determinations.

The reproducibility of the assay was determined by analyzing six times two samples of masseter muscle and was found to produce relatively low and high concentrations of HNE following Fe^{++} stimulation. The values found for mean (SE) and coefficent of variation (CV) were 3.90 (0.16) nmol/g and 9.7% in the low sample and 51.3 (2.34) nmol/g and 11.1% in the high sample.

The assay has been used to measure "peroxidizability" of liver and various muscle samples from α-tocopherol deficient and supplemented pigs and cattle; the results of these studies will be published in full elsewhere. In brief, however, pigs fed a diet depleted of α-tocopherol for 7 mon had heart α-tocopherol and induced HNE concentrations of 7.7 (0.9) and 14.2 (1.5) nmol/g, respectively. These values were significantly different ($P < 0.001$) from those observed in control animals fed the same diet, but supplemented with α-tocopherol. In those animals the heart α-tocopherol and HNE concentrations were 32.5 (2.6) and 1.8 (0.2) nmol/g, respectively. The mean concentration of HNE induced in the homogenate from the deficient group was approximately 8 times higher than that from the sufficient group. This finding indicates that heart muscle from the α-tocopherol sufficient group was much better protected against possible free radical attack than that from the α-tocopherol deficient group.

We have also found that the differences in Fe^{++}-induced HNE have generally been greater than in ascorbate-induced TBARS between α-tocopherol deficient and sufficient tissues (data not shown), suggesting that HNE may be a better indicator of "peroxidizability" than TBARS. The assay using LC/MS is relatively simple to perform. Only one derivatization step is required, as opposed to two for the GC/MS assays (10,11) where a trimethylsilyl derivative is prepared after the PFB derivative. The detection limit of the LC/MS is about 6 pmol injected on-column. This equates to about 640 pmol/g for tissue samples. This is not as sensitive as GC/MS assays using GC with negative ion chemical ionization, with detection limits about 10 times higher. The sensitivity is, however, sufficient to measure the levels of induced HNE in tissue samples, and we have not found

it necessary to try to improve on this. Deuterated HNE could be used as an internal standard, if available. The synthesis of deuterated HNE has been described (10), and its use should further increase the reproducibility of the assay. However, we have already shown (28) that a dedicated LC/MS system, such as that used in this assay, is capable of good reproducibility with a typical coefficient of variation of 3.6% between standard injections, without the use of an internal standard.

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LETTER TO THE EDITOR

Zinc and Red Cell Fatty Acid Composition

Dear Sir:

The paper by Driscoll and Bettger (1) on the effects of dietary zinc deficiency on red cell lipid and essential fatty acid (EFA) composition deserves comment with respect to extrapolation of the data to general concepts regarding the role of zinc in EFA metabolism. The dietary methodology and results in this paper agree quite closely with those of Kramer *et al.* (2) who also concluded that zinc deficiency *per se* has no significant effect on EFA composition beyond that caused by reduced food intake. The implication in both these papers is that zinc is probably not involved in Δ6 or Δ5 desaturation.

While the Δ6 desaturase is now known to be an iron-containing enzyme (3), zinc may be involved in determining overall desaturase activity by influencing electron availability from the electron donors NADH or NADPH (4). If zinc were to be involved in arachidonic acid synthesis, one would expect lower levels of arachidonic acid synthesis during zinc deficiency, as many studies have reported (5; reviewed in ref. 4), but higher arachidonic acid levels after zinc supplementation. In fact, this is the case both in animal models with normal food and nutrient intake (6,7) and in human disease; in acrodermatitis enteropathica (genetic defect impairing zinc absorption), dietary zinc supplementation significantly increases both arachidonic acid and docosahexaenoic acid in serum phospholipids (8,9). We have also recently shown that dietary zinc supplementation raises the mol% of arachidonic acid in serum phospholipids in Wilson's disease (controls, 12.6 ± 2.4%, n = 19; Wilson's disease before zinc, 10.9 ± 2.4%, n = 10; Wilson's disease after zinc, 12.7 ± 2.2%, n = 36; $P < 0.01$). Thus, the effect of zinc on arachidonic acid metabolism may be indirect, but becomes clearly apparent under a variety of circumstances.

Zinc deficiency studies in rats need not involve decreased food intake if the diet is not excessively deficient in zinc, *e.g.*, about 3 mg/kg in the diet *vs.* the < 0.3 mg/kg reported by Driscoll and Bettger (1) and Kramer *et al.* (2). Furthermore, if the low zinc diet is fed after the post-weaning growth spurt, the fatty acid changes induced by low zinc intake are still seen without any impairment in food intake (6).

The observation by Driscoll and Bettger (1) that free plasma arachidonic acid is normalized in food restricted rats refed zinc supports the majority of data which indicates a specific but undefined role of zinc in arachidonic acid synthesis. The fact that differences in red cell fatty acid composition between zinc deficient and zinc sufficient-restricted fed rats were for the most part minimal, appears to be more a function of the model than of the absence of a role of zinc in arachidonic acid synthesis.

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ARTICLE

Elongation Predominates over Desaturation in the Metabolism of 18:3n-3 and 20:5n-3 in Turbot (*Scophthalmus maximus*) Brain Astroglial Cells in Primary Culture

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The origin of docosahexaenoic acid (DHA, 22:6n-3) that accumulates in turbot brain during development was investigated by studying the incorporation and metabolism *via* the desaturase/elongase pathways of [1-^{14}C]-labelled polyunsaturated fatty acids (PUFA) in primary cultures of brain astrocytic glial cells. There was little specificity evident in the total incorporation of PUFAs into the turbot astrocytes. However, specificity was apparent in the distribution of the various PUFAs among the individual lipid classes. In particular, there was very specific incorporation of [^{14}C]arachidonic acid (AA, 20:4n-6) into phosphatidylinositol balanced by a lower incorporation of this acid into total diradyl glycerophosphocholines. [^{14}C]-Linolenic acid (LNA, 18:3n-3) and [^{14}C]eicosapentaenoic acid (EPA, 20:5n-3) were metabolized *via* the desaturase/elongase pathways to a significantly greater extent than [^{14}C]linoleic acid (18:2n-6) and [^{14}C]AA. The turbot astrocytes expressed very little Δ5 desaturase activity and only low levels of Δ4 desaturation activity. Although the percentages were small, approximately 4–5 times as much labelled DHA was produced from [^{14}C]EPA compared with [^{14}C]LNA. However, it was concluded that very little DHA in the turbot brain could result from the metabolism of LNA and EPA in astrocytic glial cells.
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During development, mammalian brains and retinas accumulate docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA) (1) so that in adults neural tissues are characteristically rich in these specific polyunsaturated fatty acids (PUFA) (2). Studies with dietary deprivation of n-3 PUFA have shown that DHA is tenaciously retained in neural tissue (3), taking at least two generations to be significantly depleted (4–7). Reduced brain and retinal DHA content is associated with altered physiological parameters and functional defects, such as reduced visual acuity and impaired learning ability (4–7), suggesting a critical and probably essential role for DHA in neural tissue development and function (8,9).

Abbreviations: AA, 5,8,11,14-arachidonic acid (20:4n-6); ANOVA, analysis of variance; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CL, cardiolipin; CPL, total diradyl glycerophosphocholines; DHA, 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3); DMEM, Dulbecco's modification of Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EPA, 5,8,11,14,17-eicosapentaenoic acid (20:5n-3); EPL, total diradyl glycerophosphoethanolamines; GC, gas chromatography; HBSS, Hank's balanced salt solution; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; LA, 9,12-linoleic acid (18:2n-6); LNA, 9,12,15-linolenic acid, α-linolenic acid (18:3n-3); PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; TN, total neutral lipids; TP, total polar lipids.

Fish neural tissues are also rich in DHA, with DHA to eicosapentaenoic acid (20:5n-3, EPA) ratios in the glycerophospholipids far exceeding those of most non-neural tissues (10,11). Although EPA levels are greater than those of AA in fish neural tissues (10), the selective location of AA in phosphatidylinositol (PI) combined with AA generally being the main eicosanoid precursor in many fish tissues, including brain cells (10–12), has implicated a similarly important role for AA in fish as in mammals. Recently, we have shown that DHA is rapidly and specifically accumulated in brains from post-larval turbot, *Scophthalmus maximus*, upon weaning from a diet low in DHA to one high in DHA (13,14). Prior to weaning, the turbot brains displayed low levels of DHA despite adequate dietary provision of both α-linolenic acid (18:3n-3, LNA) and EPA (13), suggesting that preformed DHA was required by turbot brains during development. However, biochemical studies on isolated mixed brain cells from juvenile turbot showed that there was no selectivity between [1-^{14}C]LNA and [1-^{14}C]DHA in the uptake into the cells (15). Furthermore, over 20% of [1-^{14}C]EPA incorporated into the mixed brain cells was chain elongated with up to 10% desaturated to DHA within 24 h (15).

The origin of neural tissue DHA during development in mammals is unclear. In rats, it has been shown that DHA was produced from intracranially-injected ^{14}C-labelled LNA (16), but other evidence has suggested that much of the DHA in mammalian brains results from accumulation of preformed DHA supplied by the liver (17,18). Studies using primary cultures of specific cell types from rat brain have recently shown that DHA and AA were produced from LNA and linoleic acid (18:2n-6, LA), respectively, in astrocytes, but not in neurons (19).

In the present study, we investigated the incorporation and metabolism *via* the desaturase/elongase pathways of [1-^{14}C]-labelled LA, LNA, AA and EPA in primary cultures of astrocytic glial cells from turbot. Our primary aim was to determine if astrocytes could contribute to the accumulation of DHA, particularly from EPA, during development in turbot brain. We have shown previously that an established turbot cell line, originally prepared from fin tissue, appeared to lack Δ5 desaturase activity (20–22). Therefore, we wished to determine if the primary cultures of brain astrocytes also had incomplete pathways for the desaturation and chain elongation of LA and LNA.

MATERIALS AND METHODS

Materials. Dulbecco's modification of Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), glutamine, antibiotics, fetal calf serum and trypsin/ethylenediaminetetraacetic acid (EDTA) were all obtained from Northumbria Biologicals Ltd. (Northumberland, United

Kingdom). [1-^{14}C]PUFA (all 50–55 mCi/mmol and 99% pure) were obtained from NEN, DuPont (U.K.) Ltd. (Stevenage, United Kingdom). Sodium selenite, insulin, transferrin, triiodothyronine, poly-D-lysine, butylated hydroxy toluene (BHT), fatty acid-free bovine serum albumin (BSA) and silver nitrate were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom). Mouse monoclonal antiglial fibrillary acidic protein and F(ab')$_2$ rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate were from Serotec (Oxford, United Kingdom). Rhodamine-phalloidin was a gift from Dr. R. Wilson (University of Stirling, Scotland, United Kingdom). Thin-layer chromatography (TLC) (20 × 20 cm × 0.25 mm) and high-performance thin-layer chromatography (HPTLC) (10 × 10 cm × 0.15 mm) plates, precoated with silica gel 60 (without fluorescent indicator), were obtained from Merck (Darmstadt, Germany). All solvents were high-performance liquid chromatography (HPLC) grade and were obtained from Rathburn Chemicals (Walkerburn, Scotland, United Kingdom).

Experimental animals. Turbot, *Scophthalmus maximus* (~500 g, and 7–8 mon old) were obtained from a commercial mariculture station (Golden Sea Produce, Hunterston, Scotland, United Kingdom). Fish were maintained in 2-m circular tanks supplied with recirculating, filtered sea water in the aquarium at Stirling University for between 2 and 4 wk during which period they were starved. Water temperature was 10 ± 2°C.

Preparation of primary cultures of turbot astroglial cells. Primary cultures of turbot astroglial cells were prepared by modification of methods used previously to prepare astrocytes from rainbow trout brain (23,24). Fish (four per experiment) were killed by severing the spinal cord immediately posterior to the brain. Entire brains were removed into ice-cold HBSS (Ca^{2+}- and Mg^{2+}-free) supplemented with 1.75% NaCl (HBSS/NaCl) and the meninges carefully dissected away. Brains were transferred into fresh, ice-cold HBSS/NaCl and were finely chopped. Brain cells were dissociated by sequential sieving through nylon gauzes of 190, 100 and 30 μm pore sizes essentially as described by McCarthy and de Vellis (25). The suspension was centrifuged at 300 × *g* for 10 min at 4°C and the pellet resuspended in DMEM containing 4 mM glutamine, antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin), 0.35% NaCl and further supplemented with 30 nM sodium selenite, 5 μg/mL of insulin, 50 μg/mL of transferrin, 30 nM triiodothyronine and 10% fetal calf serum. The cells were seeded into 75-cm^2 plastic tissue culture flasks (Gibco-Nunc) pretreated with polylysine. Cultures were incubated for four weeks at 22°C with the medium replaced on days 4, 14 and 24. After four weeks the cultures were well established and the incubation temperature was lowered to 15°C, which is approximately the upper limit of the ambient temperature range that turbot normally experience in United Kingdom waters, and incubation continued for a further two weeks without any medium change. The cells grow very slowly at 15°C, and therefore the cultures must be initially established at 22°C. Before use, the cultures were exposed to sheer forces to detach overlying cells (predominantly oligodendrocytes) and washed with HBSS/NaCl to remove these and dead cells (24). The resultant monolayer of cells was characterized as astrocyte-rich (~90%) by indirect immunofluorescence and dual-labelling using antiglial fibrillary acidic protein as an astrocyte marker and rhodamine-phalloidin as a general cell stain, as described in detail earlier (26).

Incubation of turbot astroglial cell cultures with [1-^{14}C]-labelled fatty acids. Medium was aspirated and the cultures washed with HBSS/NaCl. Fresh DMEM, used as above, except that it did not contain the supplements (selenite, insulin, transferrin and triiodothyronine) or serum that are required to establish the cultures, was added. Supplements are not required for the maintenance of the cell cultures and so were left out to remove their possible effects on PUFA uptake and metabolism. Serum was omitted so that the isotope was added undiluted by the fatty acids and lipids in serum. Triplicate flasks received 0.3 μCi each of either [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, [1-^{14}C]20:4n-6 or [1-^{14}C]20:5n-3, added carrier-free in ethanol (final conc., fatty acid, 0.6 μM and ethanol, 0.25%). Cultures were incubated with the isotopes for 6 d at 15°C.

Harvesting of cells and lipid extraction. The medium was aspirated, and the cultures were washed twice with 20 mL of ice-cold HBSS/NaCl containing 1% fatty acid-free BSA. The cells were harvested by trypsinization and washed further twice with 10 mL of cold HBSS/NaCl, and lipid extracted essentially according to the procedure of Folch *et al.* (27) as detailed previously (28).

Incorporation of radioactivity into total lipid and glycerophospholipid classes. After weighing, the total lipid was resuspended in chloroform/methanol (2:1, vol/vol) containing 0.01% BHT as antioxidant at a concentration of 10 mg/mL. Samples of the total lipid were added to scintillation mini-vials, the solvent was evaporated under a stream of nitrogen, 2.5 mL of Ecoscint A (National Diagnostics, Manville, NJ) were added and radioactivity was determined using a Packard 2000CA TriCarb liquid scintillation analyzer (Downers Grove, IL). Samples of total lipid (50 μg) were applied in 1-cm streaks onto HPTLC plates, and the glycerophospholipid and other polar lipid classes were separated using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) (29). The lipid classes were vizualized by brief exposure to iodine vapor and marked, and the iodine removed under vacuum. Individual glycerophospholipid classes were scraped into scintillation mini-vials, and radioactivity was determined as above.

Incorporation of radioactivity into PUFAs. Fatty acid methyl esters were prepared from total lipid by acid catalyzed transmethylation at 50°C for 16 h (30) and were extracted and purified as described previously (10). Methyl esters were separated by a combination of argentation-TLC and radio-gas chromatography, and the identity of fatty acid methyl esters and the radioactivity in the individual methyl ester fractions determined as described previously in detail (22,24). The identity of 24:5n-3 was confirmed by gas chromatography/mass spectrometry. All solvents contained 0.01% BHT as antioxidant.

Statistical analysis. All results are presented as means ±SD (n = 3). The data were checked for homogeneity of the variances by the Bartlett test and, where necessary, the data were arcsin-transformed before further statistical analysis. Data for the incorporation of radioactivity into total lipid and individual lipid classes were analyzed by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's multiple comparison test. Differences between the mean recoveries of radioactivity from

[^{14}C]18:3n-3 and [^{14}C]20:5n-3 in specific fatty acid fractions, compared with the recoveries of radioactivity from [^{14}C]18:2n-6 and [^{14}C]20:4n-6, respectively, in the homologous fatty acid fractions (*i.e*, recovery of radioactivity from [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 in the 22:6n-3 fraction was compared with the recovery of radioactivity from [^{14}C]18:2n-6 and [^{14}C]20:4n-6, respectively, in the 22:5n-6 fraction) were analyzed by the Student's *t*-test. Differences were reported as significant if $P < 0.05$.

RESULTS

The ^{14}C-labelled PUFAs were incorporated into the total lipid in amounts ranging from 4.10 to 4.77 pmol/μg total lipid (Table 1). The only significant difference between the four different PUFAs was that the incorporation of [^{14}C]AA was significantly less than the incorporation of [^{14}C]EPA (Table 1).

The incorporation of the C_{20} PUFA into total polar classes was generally greater than the incorporation of the C_{18} PUFA into total polar classes, although the difference between [^{14}C]LA and [14C]EPA was not statistically significant (Fig. 1). The percentage incorporation into polar lipid classes was lowest, and therefore the percentage incorporation into neutral lipids was highest, with [^{14}C]LNA. There were several significant differences between the [^{14}C]PUFA in their incorporation into the individual glycerophospholipid classes. The most striking was the significantly greater incorporation of [^{14}C]AA into PI balanced by significantly lower incorporation of this acid into diradyl glycerophosphocholines (CPL) (Fig. 1). Both ^{14}C-labelled C_{18} PUFA were incorporated into the phosphatidic acid/cardiolipin (PA/CL) fraction to a significantly greater extent than the ^{14}C-labelled C_{20} PUFA (Fig. 1).

Significantly more of the incorporated [^{14}C]LNA was metabolized *via* the desaturase/elongase pathway compared to [^{14}C]LA (Table 2). The majority of the metabolized [^{14}C]LNA was chain elongated to 20:3n-3, 22:3n-3 and 24:3n-3, whereas only 4.7% of the incorporated [^{14}C]LA was elongated to 20:2n-6, with no labelled 22:2n-6 and 24:2n-6 being detected (Table 2). Approximately equal amounts of radioactivity (~6–7%) from [^{14}C]LA and [^{14}C]LNA were found in the products of Δ6 desaturation. Radioactivity was detected in 22:4n-3, whereas no radioactivity was found in trienoic n-6 PUFA of chain length greater than C_{20}. Significantly more radioactivity from [^{14}C]LA, compared to [^{14}C]LNA, was found in the products of Δ5 desaturation, both 20:4n-6 and 22:4n-6. In contrast, a small percentage of radioactivity from [^{14}C]LNA was found in the DHA fraction, whereas no radioactivity from [^{14}C]LA was detected in 22:5n-6 (Table 2).

A significantly greater percentage of the incorporated [^{14}C]EPA was metabolized by chain elongation and desaturation compared to [^{14}C]AA (Table 3). The majority of the metabolized [^{14}C]EPA was chain elongated to 22:5n-3 (14.7%) and 24:5n-3 (8.7%) with less than 2% desaturated to DHA. The majority of the [^{14}C]AA metabolized was chain elongated to 22:4n-6 with only a trace desaturated to 22:5n-6 (Table 3). There was no labelling in elongation products of AA with chain length greater than C_{22}. In turbot astrocytes labelled with [^{14}C]EPA, small percentages of radioactivity were detected in less unsaturated fatty acid fractions. No labelling was detected in less unsaturated fatty acid fractions in turbot astrocytes labelled with [^{14}C]AA (Table 3).

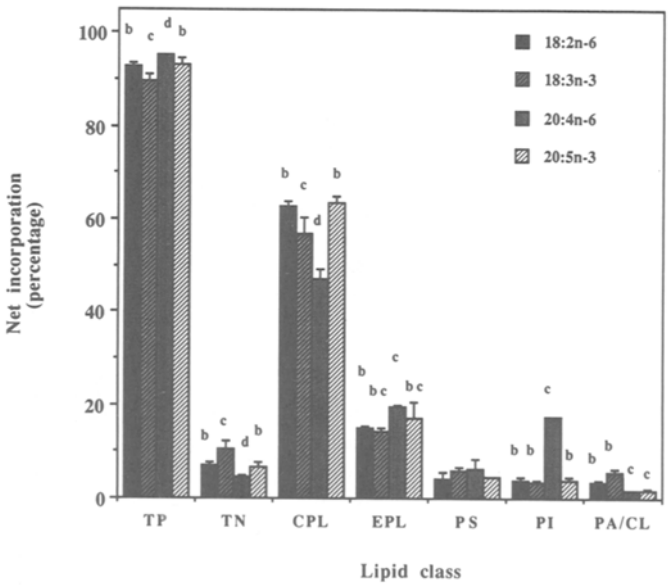

FIG. 1. Net incorporation of [1-^{14}C]-labelled fatty acids into lipid classes after 6 d incubation in turbot astroglial cells in primary culture. Results are means ± SD of three experiments. Statistical analysis is described in Materials and Methods. Values of columns within each grouping with different superscript letters are significantly different ($P < 0.05$). CPL, total diradyl glycerophosphocholines; EPL, total diradyl glycerophosphoethanolamines; PA/CL, phosphatidic acid/cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine; TN, total neutral lipid classes; TP, total polar lipid classes.

TABLE 1

Net Incorporation of [1-^{14}C]-Labelled Fatty Acids into Total Lipid from Turbot *(Scophthalmus maximus)* Astroglial Cells in Primary Culture[a]

[1-^{14}C]Fatty acid	Incorporation (pmol/μg total lipid)
[1-^{14}C]18:2n-6	4.77 ± 0.46^{bc}
[1-^{14}C]18:3n-3	4.34 ± 1.04^{bc}
[1-^{14}C]20:4n-6	4.10 ± 0.02^{b}
[1-^{14}C]20:5n-3	4.76 ± 0.15^{c}

[a]Results are means ± SD of three experiments. Cells were incubated with isotopes for 6 d at 15°C. Statistical analysis is described in Materials and Methods. Values with different superscript letters are significantly different ($P < 0.05$).

DISCUSSION

This paper is the first report of a primary culture of monolayers of any brain cell type from a marine fish. The methods used were modified from those used previously to culture astroglial cells from rainbow trout, *Oncorhynchus mykiss* (23,24). For the turbot astrocytes, all media and salt solutions were supplemented with NaCl. The main difference in the procedures was that the turbot astrocytes showed a much slower growth rate compared to the trout astrocytes, taking approximately twice as

TABLE 2

Metabolism of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 by the Desaturase/Elongase Pathways in Turbot *(Scophthalmus maximus)* Astroglial Cells in Primary Culture[a]

Fatty acid fraction	Polyunsaturated fatty acid added	
	[1-^{14}C]18:2n-6	[1-^{14}C]18:3n-3
18:2n-6/18:3n-3	84.0 ± 2.0	71.1 ± 7.3[b]
20:2n-6/20:3n-3	4.7 ± 0.6	13.7 ± 1.3[b]
22:2n-6/20:3n-3	n.d.	6.6 ± 3.9[b]
24:2n-6/20:3n-3	n.d.	2.5 ± 1.5[b]
18:3n-6/18:4n-3	2.8 ± 0.2	3.4 ± 1.4
20:3n-6/20:4n-3	4.6 ± 3.5	2.2 ± 0.1
22:3n-6/22:4n-3	n.d.	0.1 ± 0.1
24:3n-6/24:4n-3	n.d.	n.d.
20:4n-6/20:5n-3	2.5 ± 0.5	0.2 ± 0.1[b]
22:4n-6/22:5n-3	1.6 ± 0.8	0.1 ± 0.1[b]
24:4n-6/24:5n-3	n.d.	n.d.
22:5n-6/22:6n-3	n.d.	0.4 ± 0.2[b]

[a]Results are expressed as percentages of total radioactivity recovered and are means ± SD of three experiments. Cells were incubated with isotopes for 6 d at 15°C. n.d., not detected. Values for the recovery of radioactivity from added [^{14}C]18:3n-3 in each fatty acid fraction were compared with the recovery of radioactivity from [^{14}C]18:2n-6 in the homologous fatty acid fractions.

[b]The differences were statistically significant ($P < 0.05$).

TABLE 3

Metabolism of [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 by the Desaturase/Elongase Pathways in Turbot *(Scophthalmus maximus)* Astroglial Cells in Primary Culture[a]

Fatty acid fraction	Polyunsaturated fatty acid added	
	[1-^{14}C]20:4n-6	[1-^{14}C]20:5n-3
18:2n-6/18:3n-3	n.d.	0.8 ± 0.1[b]
20:2n-6/20:3n-3	n.d.	
22:2n-6/22:3n-3	n.d.	n.d.
24:2n-6/24:3n-3	n.d.	n.d.
18:3n-6/18:4n-3	n.d.	1.8 ± 0.8[b]
20:3n-6/20:4n-3	n.d.	
22:3n-6/22:4n-3	n.d.	n.d.
24:3n-6/24:4n-3	n.d.	n.d.
20:4n-6/20:5n-3	94.9 ± 1.1	73.0 ± 8.0[b]
22:4n-6/22:5n-3	5.0 ± 1.1	14.7 ± 5.9[b]
24:4n-6/24:5n-3	n.d.	8.7 ± 0.8[b]
22:5n-6/22:6n-3	0.1 ± 0.1	1.9 ± 0.6[b]

[a]Results are expressed as percentages of total radioactivity recovered and are means ± SD of three experiments. Cells were incubated with isotopes for 6 d at 15°C. n.d., not detected. Values for the recovery of radioactivity from added [^{14}C]20:5n-3 in each fatty acid fraction were compared with the recovery of radioactivity from [^{14}C]20:4n-6 in the homologous fatty acid fractions.

[b]The differences were statistically significant ($P < 0.05$).

long to reach 75% confluence, the point at which the isotopes were added. With the longer growth period it was necessary to change the medium more often than was required with the trout astrocyte cultures.

The incorporation of [^{14}C]AA into the turbot astrocytes was significantly less than the incorporation of [^{14}C]EPA, but this was primarily due to the very low deviation (<0.5% of the mean) found in the AA data compared to the variances in the data for the other three PUFA, which ranged from 3 to 24% in the present study. A wide range of variances has consistently been observed in previous studies measuring the incorporation of radiolabelled PUFA into cultured fish cells, with reported variances ranging from 2.3 to 22.5% (24), 1 to 41.3% (22) and 15.3 to 39.3% (31). There was no difference in the incorporation into total lipid between [^{14}C]labelled LA, LNA, AA and EPA in trout astrocytes (24) or established cell cultures from trout, Atlantic salmon *(Salmo salar)* and turbot (22). In brain cell suspensions from turbot, there was no significant difference in the level of incorporation into total lipid between [^{14}C]LNA and [^{14}C]DHA (15). Therefore, the data in the present study are consistent with data obtained previously in fish astrocyte cultures and turbot cells generally. In contrast, in trout brain cell suspensions, the incorporation of ^{14}C-labelled C_{20} PUFA into total lipid tended to be lower than that of ^{14}C-labelled C_{18} PUFA, with the incorporation of [^{14}C)AA generally exceeding that of [^{14}C]EPA (31).

Between 90 and 95% of the total radioactivity from the four ^{14}C-labelled PUFAs incorporated into turbot astrocytes was esterified into polar lipid classes. An almost identical situation was found in trout astrocytes where 90 to 96% of the same four PUFAs were incorporated into total phospholipids (24). Similarly, between 83 and 95% of incorporated ^{14}C-labelled LA and LNA and between 95 and 98% of incorporated ^{14}C-labelled AA and EPA were esterified into phospholipids in trout, salmon and turbot cell lines (22). In contrast, in suspensions of isolated mixed brain cells from both trout and turbot, the greater percentages of the incorporated ^{14}C-labelled PUFA were generally found in the neutral lipid fractions, particularly with C_{20} and C_{22} PUFA (15,31). The distribution of radiolabel from [^{14}C]LA, LNA and EPA among the glycerophospholipid classes observed in the present study, with the rank order being CPL > EPL > PS and PI, has been observed in previous studies on fish brain cells and cell cultures (15,22,24,31), as has the specificity for incorporation of [^{14}C]AA into PI (22,24,31).

The n-3 PUFA, LNA and EPA were clearly preferred to the n-6 PUFA, LA and AA, as substrates for the desaturase/elongase pathways in turbot astrocytes. A similar preference was also observed in trout astrocyte cultures (24) and trout, salmon and turbot cell lines (22) in experiments of a similar duration (6–7 d incubation with isotope). In short-term experiments over 24 h in trout brain cell suspensions, there was no difference in the extent of metabolism between LA and LNA, although there was significantly more EPA metabolized *via* desaturation and elongation as compared to AA (31). The turbot astrocytes were unique in the extent to which elongation predominated over desaturation, particularly with LNA and EPA. The production of significant amounts of labelled 22:3n-3 and 24:3n-3, and some 22:4n-3 from [^{14}C]LNA and labelled 24:5n-3 from [^{14}C]EPA was not observed previously in studies in trout astrocytes (24) or mixed brain cells (31) or trout and salmon cell lines (22). However, radioactivity was detected in apparently longer chain metabolites, although at much lower levels than the

present study, in turbot fin cells incubated with labelled LNA and EPA for 7 d, but the identity of these was not confirmed in that study. In contrast, turbot brain cell suspensions incubated with LNA and EPA for 24 h showed only traces of radioactivity in the C_{22} and C_{24} PUFAs mentioned above (15). Therefore, the production of these longer-chain metabolites appears to be a characteristic of turbot cells, in particular astrocytes, but their biosynthesis may be more time-dependent than the normally observed desaturase products.

In mixed brain cell suspensions from trout and turbot, significant percentages of EPA were desaturated to DHA in 24 h. In trout brain cells, between 4.3 and 9.3% of label from [^{14}C]EPA was recovered in DHA, depending upon culture temperature (31), and for turbot mixed brain cells the equivalent figures were 7.1 to 10.0%, depending upon the age of the fish (15). In contrast, in the astrocyte cultures from turbot brain, less than 2% of the radioactivity from [^{14}C]EPA was recovered in DHA after 6 d incubation, and for trout astrocytes the corresponding figure was between 3.2 and 5.3%, depending upon culture temperature (24). Therefore in fish, particularly turbot, the enriched astrocyte cultures were less efficient in conversion of EPA to DHA as compared to mixed brain cell suspensions. This contrasts with the situation in neonatal rat brain astrocyte cultures which were very active in the production of DHA (19).

The above data indicate that fish astrocytes have rather low levels of Δ4 desaturation activity. This activity is often lost in cultured cells, apparently by the process of culturing itself (32). The elongation of LA to 20:2n-6 and LNA to 20:3n-3, the so-called "dead-end" pathway, is often increased in cells lacking Δ4 desaturation activity, and that is the case with the turbot astrocytes. However, recent evidence has suggested that there may not be an actual Δ4 desaturase enzyme, and that desaturation of 22:5n-3 to DHA may occur *via* elongation of 22:5n-3 to 24:5n-3, and Δ6 desaturation of 24:5n-3 to produce 24:6n-3 in the microsomes, followed by peroxisomal chain shortening of 24:6n-3 to DHA (33). Although a significant percentage of label from [^{14}C]EPA was recovered in 24:5n-3 in the turbot astrocytes, there was no evidence for labelled 24:6n-3, despite the presence of reasonable Δ6 desaturase activity toward LA and LNA.

Nutritional studies suggested that both turbot larvae and post-larvae (<1-mon-old) were unable to convert dietary EPA to DHA, in the brain at least (13,14). However, biochemical studies showed that mixed brain cells from older fish (1- and 4-mon-old) desaturated and elongated significant amounts of EPA to DHA (15). The activity was higher in the 4-mon-old fish, perhaps suggesting increasing Δ4 desaturation activity with age (15). In the present study, astrocytes from fish, approximately 8-mon-old, had very little Δ4 desaturation activity. Therefore, the data from the present study indicates that only a small percentage of the Δ4 desaturation activity observed in the mixed brain cell suspensions could be attributed to astroglial cells.

The turbot astrocytes lack, or express only very low, Δ5 desaturase activity. This is consistent with data obtained from *in vivo* injection studies (34,35) and *in vitro* cell studies (15,20,21). It was surprising that in the astrocytes significantly more label from [^{14}C]LA was recovered in n-6 tetraenes compared to the recovery of label from [^{14}C]-LNA in n-3 pentaenes (Table 2). This was not observed in the previous studies in turbot cells (15,20,21) and, in general, all the desaturase activities in fish cells are more active toward n-3 PUFA substrates (20,24,31,36,37), as previously reported for mammalian Δ6 desaturase activity (38).

In conclusion, astrocytic glial cells from turbot brain were similar to the other turbot tissues studied in that they expressed very little Δ5 desaturase activity. In addition, the cultured astrocytes had relatively low Δ4 desaturation activity. Consequently, we conclude that very little of the DHA that accumulates during development in the turbot brain could result from the metabolism of LNA and EPA in astrocytic glial cells.

A lack, or very low levels, of Δ5 desaturase activity appears to be a characteristic of marine fish species (11,15,20,34). Therefore, it is probable that DHA in the brains of marine fish in general cannot be derived from the metabolism of LNA *in situ*. In this respect, marine fish are similar to terrestrial carnivores such as the cat (39), and, in any case, like terrestrial carnivores, marine fish in the wild will probably have little dietary input of LNA. However, the turbot is also highly carnivorous (11,13,14), and so the study of a herbivorous marine fish is required to confirm the hypothesis for marine fish in general. How much DHA in the brains of marine fish is derived from the metabolism of EPA requires more extensive study of Δ4 desaturation in fish tissues, including the brain, particularly in light of the recent work suggesting that there may not be a specific Δ4 desaturase enzyme (33). Larval and early juvenile stages of marine fish should also be studied as the nutritional studies with cultured species suggest that it is at these times of rapid growth that dietary DHA may be limiting in live feed diets (13,14). However, the same situation may not apply to freshwater fish species which do not lack Δ5 desaturase and so are able to desaturate and elongate C_{18} essential fatty acids to EPA and DHA (11,34). Therefore, it is possible that the *in situ* metabolism of both LNA and EPA may contribute to brain DHA in freshwater fish. In some support of this we have shown that the production of [^{14}C]-labelled DHA from [^{14}C]LNA and [^{14}C]EPA in rainbow trout astrocytes (24) was at least twice the level of [^{14}C]-labelled DHA produced from [^{14}C]LNA and [^{14}C]EPA in the present study.

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Detection of [U-^{13}C]Eicosapentaenoic Acid in Rat Liver Lipids Using ^{13}C Nuclear Magnetic Resonance Spectroscopy

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Fatty acid carbons are well-resolved in ^{13}C nuclear magnetic resonance (NMR) spectra of lipid extracts, but application of this methodology to the metabolism of ^{13}C-labelled fatty acids has not yet been reported. In the present study, ^{13}C NMR was used to monitor the presence of 98% [U-^{13}C]eicosapentaenoic acid (EPA) in liver and carcass lipids 24 h after it had been injected into the stomach of a rat. Natural abundance ^{13}C NMR spectra of liver total fatty acid extracts were obtained from four control rats for comparison. At 24 h post-injection, quantitative high resolution ^{13}C NMR showed ^{13}C enrichment in liver fatty acid extracts was present mainly at olefinic and at the n-1 to n-4 carbons, but ^{13}C signal intensities for C-1 to C-4 of [U-^{13}C]EPA were markedly reduced or absent. Small ^{13}C resonances, possibly indicative of some ^{13}C incorporation into docosahexaenoic acid and saturated or monounsaturated fatty acids, were present in spectra of liver fatty acids. Liver and carcass fatty acid composition was similar in both the controls and the EPA-injected rat, suggesting little accumulation of the injected [U-^{13}C]EPA after 24 h. We conclude that the carbon-specific data provided by ^{13}C NMR of lipid extracts may be useful in monitoring the fate of individual carbons during tracer studies using ^{13}C-labelled fatty acids.
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^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) is a useful technique for metabolic studies because of the wide spectral dispersion of ^{13}C signals (1–4) and because of the potential to monitor the metabolism of ^{13}C-labelled compounds as tracers in various biochemical pathways. The use of ^{13}C NMR to monitor incorporation of ^{13}C from [2-^{13}C]acetate into rat liver fatty acids has been reported (5), and fatty acid carbon assignments in ^{13}C NMR spectra are well-established (6–10), but this methodology has not yet been applied to tracer studies of ^{13}C-labelled fatty acids.

Eicosapentaenoic acid (20:5n-3; EPA) has a pivotal role in long-chain fatty acid metabolism as a precursor in the synthesis of its structurally more important homologue, docosahexaenoic acid (22:6n-3; DHA). It is also an important modulator of the synthesis and metabolism of eicosanoids derived from arachidonic acid (20:4n-6) (11,12). We have used high resolution ^{13}C NMR to detect [U-^{13}C]EPA in total lipid extracts of liver. [U-^{13}C]-labelled long chain fatty acids are not generally available, so the opportunity to study the metabolism of a single 89-mg sample of [U-^{13}C]EPA from an EPA-producing microalgae consuming ^{13}C-labelled glucose was unique. As the present results are based on only one experimental animal (and four controls), they must be considered as preliminary results.

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Abbreviations: DHA, docosahexaenoic acid (22:6n-3); EPA, eicosapentaenoic acid (20:5n-3); GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; TMSS, tetrakis(trimethylsilyl)silane.

MATERIALS AND METHODS

Animals. Male Wistar rats (250–300 g) were housed individually and consumed rodent chow and tap water *ad libitum.* The rat receiving the [U-^{13}C]EPA was injected with the anesthetic, Inactin (100 mg/kg *i.p.*), and a nasogastric tube was inserted to inject the bolus of [U-^{13}C]-EPA directly into the stomach. Control rats (n = 4) were also anesthetized but received no injection. Only one rat was injected with [U-^{13}C]EPA because only a single 89-mg sample was available and no previous studies of this type have been reported from which the dose required for detection by ^{13}C NMR could have been determined.

Lipid extraction. To estimate the whole body fatty acid content of EPA and other n-3 fatty acids in the [U-^{13}C]-EPA-injected rat compared with uninjected controls, all the rats were killed and the liver and carcass (whole body − liver) were weighed and homogenized. The total liver and carcass lipids were extracted into chloroform/methanol (2:1, vol/vol) (13). Lipid extracts were saponified and the free fatty acids extracted. The total fatty acid extracts were dried under nitrogen and weighed. Aliquots (80–100 mg) of each were weighed, combined with 10 mg internal standard [tetrakis(trimethylsilyl)silane (TMSS); Aldrich Chemical Co., Milwaukee, WI] and dissolved in 500 μL of deuterated chloroform (Silanor; MSD Isotopes, Montreal, Quebec, Canada) in 5-mm NMR tubes (Wilmad Glass Co., Buena, NJ) for high resolution ^{13}C NMR (5). The resonance for TMSS is 2.67 ppm downfield relative to that of the usual chemical shift reference, tetramethylsilane at 0.00 ppm. TMSS was used as an internal standard because it is crystalline at room temperature and has a spin-lattice relaxation time of 2.56 s. Thus, with suitable NMR acquisition conditions (5) it could be used to obtain quantitative ^{13}C NMR data.

High resolution ^{13}C NMR. All high resolution ^{13}C NMR spectra were obtained using a broad band, 5-mm probe in an AM300 WB spectrometer operated at 75.4 MHz and 300 K (Bruker Canada, Milton, Ontario, Canada). Quantitative signal acquisition conditions were used, including long relaxation delay, inverse gated ^{1}H decoupling for suppression of nuclear Overhauser enhancement of ^{13}C resonances (see legend to Fig. 1 for details; also Refs. 5,10). Spectra were processed at the spectrometer or were down-loaded to an IBM AT-type computer for Fourier transformation, line-fitting of peaks (as required) and integration relative to the quantitative internal standard, TMSS (5). Peak integrals were determined using the Marquardt-Levenberg routine (NMR-286 program; Soft-Pulse Software, Guelph, Ontario, Canada).

Fatty acid analysis. Aliquots of the total fatty acid extracts of liver and carcass were methylated using 14% boron trifluoride in methanol (Sigma Chemical Co., St. Louis, MO) at 90°C for 30 min in screw-capped glass tubes under nitrogen (5). The resulting fatty acid methyl esters were separated by capillary gas-liquid chromatography (GLC) on a 30 m by 0.25 μm column (Durabond 23;

J&W Scientific, Folsom, CA) using Hewlett-Packard GLC equipment (5890A with automated sample delivery and injection; Palo Alto, CA).

Data presentation. Natural abundance ^{13}C NMR data for liver and carcass lipids from control rats have been expressed as μg ^{13}C/mg liver or carcass lipid. The lipid extracts contain mixtures of fatty acids, and ^{13}C NMR does not usually distinguish between different acyl chain lengths so carbon positions in the fatty acids have been designated from both ends of the molecule using the conventional abbreviations, "C" from the carboxyl end and "n" from the methyl end.

RESULTS

[U-^{13}C]Eicosapentaenoic acid. The main difference between ^{13}C NMR spectra of 95% pure EPA containing only natural abundance ^{13}C and 98% [U-^{13}C]-enriched EPA is the presence of multiplet peaks in the spectrum of [U-^{13}C]EPA caused by ^{13}C-^{13}C coupling due to the 90-fold ^{13}C enrichment at each carbon (Fig. 1). The ^{13}C-^{13}C splittings were either doublets (n-1 at 14.1 ppm) or mixed doublet pairs (n-2 carbon at 20.5 ppm; C-2 at 33.5 ppm; n-4 carbon at 127 ppm or n-3 carbon at 132 ppm). The multiplet at 20.5 ppm appears to be a triplet, but is actually two adjacent doublets. The ^{13}C resonance for C-2 of [U-^{13}C]EPA results in a doublet of doublets because it is split once by C-1 and is also split by C-3.

Resonances for several carbons with identical or nearly identical magnetic environments give larger, more complex peaks, *e.g.,* doubly allylic carbons at 25.5 ppm (which partially overlap with C-3 of EPA at 24.8 ppm and with C-4 of EPA at 26.5 ppm). If methylene-interrupted olefinic resonances are present (as in *cis*-polyunsaturated fatty acids), two envelope regions of olefinic resonances occur at about 128 ("inner" olefinic) and 130 ppm ("outer" olefinic). In the case of EPA and other n-3 fatty acids, the n-3 olefinic carbon resonance is affected by its close proximity to the n-1 carbon and, hence, this C=C resonance in EPA is shifted downfield from 130 to 132 ppm leaving the area at 130 ppm void of peaks (Fig. 1).

High resolution ^{13}C NMR. Part of the natural abundance ^{13}C NMR spectrum of rat liver fatty acids is shown in Figure 2B. ^{13}C spectra of the carcass extracts are not shown (the data are given later in Tables 2 and 3). For the most part, ^{13}C resonances in different n-3 fatty acids are the same or very close, but there are some unique resonances, *e.g.,* C-1 of DHA at 173.4 *vs.* 174 ppm, C-2 of EPA at 33.5 *vs.* 34 ppm and C-7 of DPA at 130.0 ppm (Table 1). Unfortunately, the C-2 resonance of EPA overlaps with C-2 of arachidonic acid, so unless arachidonic acid is present in very low amounts or EPA in high amounts, EPA itself cannot be readily distinguished in ^{13}C NMR spectra.

The peak areas in the ^{13}C natural abundance spectra of liver and carcass fatty acids have been quantitated against the integrated peak area of TMSS (Table 2). The natural abundance ^{13}C NMR spectra of liver and carcass fatty acids differed primarily at the olefinic carbons; the liver fatty acids were more polyunsaturated, hence the larger value for the ^{13}C/mg lipid at the "inner" olefinic resonances in the liver fatty acids. However, the carcass fatty acids were more monounsaturated, hence the larger value for ^{13}C content of the "outer" olefinic resonances

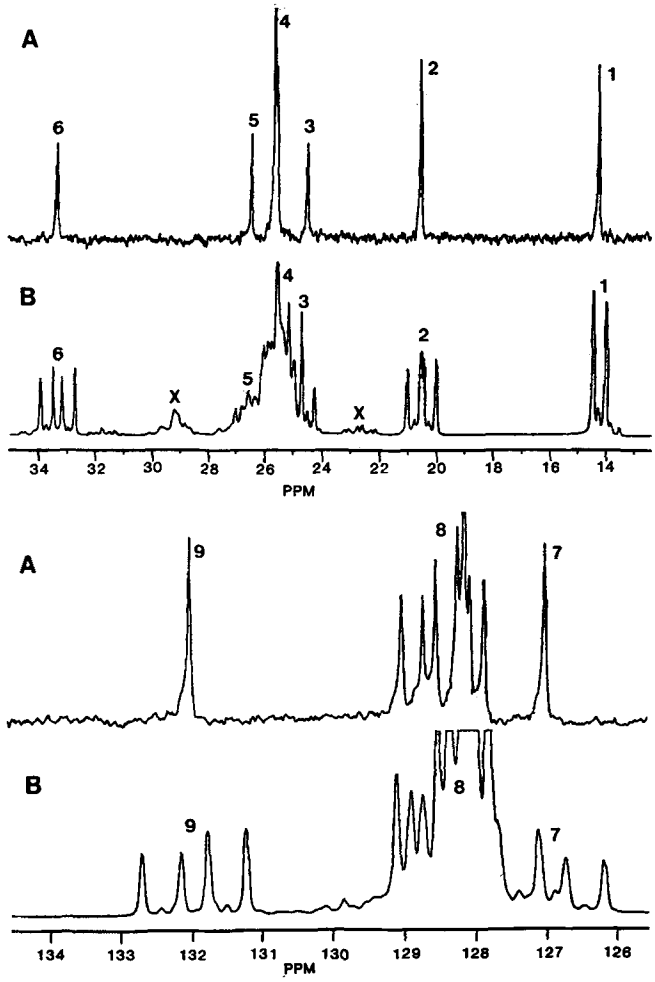

FIG. 1. Partial higher resolution ^{13}C nuclear magnetic resonance spectra of natural abundance (A), compared to 98% [U-^{13}C]eicosapentaenoic acid (B), showing the major regions of interest. Peak identification: 1, n-1 carbon; 2, n-2 carbon of n-3 fatty acids; 3, C-3 carbon; 4, doubly allylic carbons; 5, C-4 carbon; 6, C-2 carbon; 7, n-4 carbon of n-3 fatty acids; 8, "outer" olefinic carbons; 9, n-3 carbon of n-3 fatty acids; X, minor fatty acid impurities. Note that for [U-^{13}C]-eicosapentaenoic acid, most of the resonances are multiplets centered on the equivalent resonance of the natural abundance eicosapentaenoic acid. Each free induction decay comprised 1000 sequentially added scans. A sweep width of 17,281 Hz and 16K data points were used. Operating parameters were: acquisition time, 0.475 s; relaxation delay, 10.0 s; and excitation pulse width, 30°.

(Table 2). "Outer" olefinic carbon resonances occur at ~130 ppm and in rat liver are mainly from C-9 and C-10 of oleic acid and C-9 and C-13 of linoleic acid. "Inner" olefinic carbon resonances occur at about 128 ppm and are mainly from C-10 and C-12 of linoleic acid, as well as the 6 "inner" olefinic carbons of arachidonic acid and the 10 "inner" olefinic carbons from DHA; monounsaturated fatty acids have no "inner" olefinic carbons. These ^{13}C NMR data are confirmed by the fatty acid data obtained by GLC for liver and carcass, which shows 37% of liver total fatty acids were polyunsaturated compared to 30% in the carcass, but 35% were monounsaturated in the carcass compared to 16% in the liver (Table 3).

^{13}C spectra of the liver fatty acid extracts from a control rat (only natural abundance ^{13}C) and from the rat

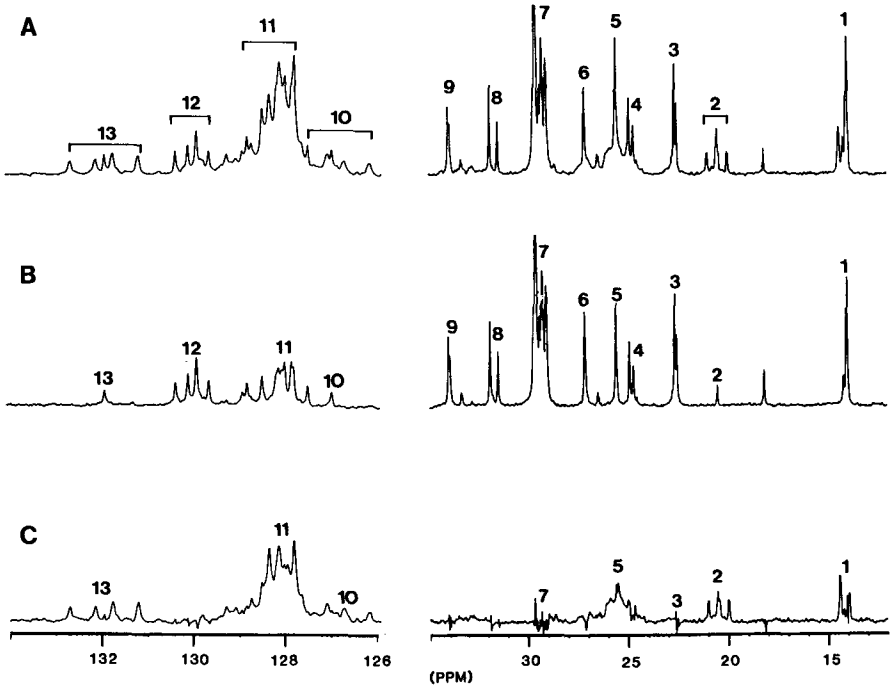

FIG. 2. Partial ^{13}C nuclear magnetic resonance (NMR) spectrum of liver total fatty acids extracted from a rat orally injected with a bolus of 98% [U-^{13}C]eicosapentaenoic acid 24 h previously (A), compared to a natural abundance ^{13}C NMR spectrum from an uninjected control rat (B), with the difference between A and B shown as C. Peak identification: 1, n-1 carbon; 2, n-2 carbon of n-3 fatty acids; 3, n-2 carbon plus C-3 of docosahexaenoic acid; 4, C-3 carbon; 5, doubly allylic carbons; 6, allylic carbons; 7, aliphatic carbons; 8, n-2 carbon; 9, C-2 carbon; 10, n-4 carbon of n-3 fatty acids; 11, "outer" olefinic carbons; 12, "inner" olefinic carbons; 13, n-3 carbon of n-3 fatty acids. Spectrometer operating conditions were as described in the legend to Figure 1, except that 5000 scans were obtained. Quantitative values shown in Table 4 are derived from Figures 2A and 2B; Figure 2C is for visual purposes only.

TABLE 1

^{13}C Nuclear Magnetic Resonances for Eicosapentaenoic Acid (EPA), Docosapentaenoic Acid (DPA) and Docosahexaenoic Acid (DHA) Relative to a Reference Standard at 0.00 ppm (adapted from Ref. 7)[a]

Carbon number	EPA	DPA	DHA
C-1	173.99	174.14	173.45
C-2	33.43	34.05	34.01
C-3	24.80	24.88	22.81
C-4	26.57	28.81	—
C-5	—	29.27	—
C-6	—	27.06	—
C-7	—	130.00	—
Doubly allylic	~25.6	~25.6	~25.6
Olefinic	128–129	128–129	128–129
n-4	127.03	127.04	127.02
n-3	132.02	132.02	132.00
n-2	20.57	20.57	20.57
n-1	14.29	14.27	14.28

[a]"C" carbons are those numbered from the carboxyl carbon; "n" carbons are those numbered from the terminal methyl carbon.

TABLE 2

Quantitative Analysis of Selected Carbons of Rat Liver and Carcass Total Fatty Acids by Natural Abundance ^{13}C Nuclear Magnetic Resonance Spectroscopy (n = 4; mean ± SD)[a]

Chemical shift (ppm)	Fatty acid carbon	μg ^{13}C/mg lipid	
		Liver	Carcass
14.1	n-1	36 ± 3	44 ± 2
25.6	Doubly allylic	22 ± 2	15 ± 2
31.5	n-3 (n-6)	9 ± 1	10 ± 2
31.9	n-3	17 ± 3	25 ± 4
34.0	C-2	20 ± 1	33 ± 4
~128	Olefinic ("inner")	58 ± 5	30 ± 7
~130	Olefinic ("outer")	23 ± 3	43 ± 5

[a]Determined relative to the peak area of a known amount of tetrakis-(trimethylsilyl)silane added as standard.

injected 24 h previously with [U-^{13}C]EPA were broadly similar as they both contained the ^{13}C signals of liver fatty acids (Fig. 2). The presence of a ^{13}C-labelled n-3 fatty acid(s) in the liver increased the ^{13}C signal intensity at several carbons of the liver, notably at the n-1 carbon (14.1 ppm), the n-2 carbon resonance of n-3 fatty acids (20.5 ppm) doubly allylic carbons (25.6 ppm) and at "inner" olefinic carbons (126–133 ppm; Fig. 2). From C-7 to the methyl terminal, the ^{13}C resonances of all n-3 fatty acids are virtually identical (Table 1) so the ^{13}C enrichment observed for these carbons does not distinguish EPA from other n-3 fatty acids which may have been synthesized from it in the 24 h after the [U-^{13}C]EPA was injected.

TABLE 3

Fatty Acid Composition (%) of the Liver and Carcass in Control Rats Compared to a Rat Orally Injected with a Bolus of [U-^{13}C]Eicosapentaenoic Acid (EPA)[a]

	Liver		Carcass	
	Control	EPA	Control	EPA
18:3n-3	0.4 ± 0.3	0.3	1.6 ± 0.3	1.0
20:5n-3	0.6 ± 0.1	0.7	0.2 ± 0.04	0.2
22:5n-3	1.1 ± 0.4	1.1	0.5 ± 0.1	0.4
22:6n-3	5.2 ± 0.4	6.2	1.1 ± 0.2	0.8
Sum n-3	7.3 ± 0.9	8.4	3.3 ± 0.3	2.4
Sum n-6	29.9 ± 2.1	29.7	27.1 ± 3.1	23.4
Sum mono	16.0 ± 2.3	15.3	34.8 ± 2.7	41.2
Sum sat	46.8 ± 3.7	46.9	34.9 ± 1.4	33.2

[a]Mean ± SD, n = 4 in controls; n = 1 in the EPA group. Sum n-3 = (18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3). Sum n-6 = (18:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6). Sum mono (monounsaturated) = (16:1n-7 + 18:1n-9 + 20:1n-9). Sum sat (saturated) = (14:0 + 16:0 + 18:0).

TABLE 4

Increase in ^{13}C Nuclear Magnetic Resonance Signal Intensity (%) at Selected Carbons in Total Fatty Acid Extracts of the Liver and Carcass of a Rat Orally Injected 24 h Previously with a Bolus of 98% [U-^{13}C]Eicosapentaenoic Acid[a]

Chemical shift (ppm)	Fatty acid carbon	^{13}C Signal intensity	
		Liver	Carcass
14.1	n-1	+83	+9
25.6	Doubly allylic	+45	−13
~128	Olefinic ("inner")	+245	+16
~130	Olefinic ("outer")	+17	+12

[a]Determined relative to the peak area of a known amount of tetrakis(trimethylsilyl)silane added as standard. Data are expressed as the increase above natural abundance ^{13}C values in control animals.

^{13}C enrichment at the C-1 to C-4 resonances of EPA were markedly reduced relative to those in the [U-^{13}C]-EPA itself (compare Figs. 1 and 2C). There were also three small ^{13}C resonances in the difference spectrum (Fig. 2C) which do not occur in [U-^{13}C]EPA itself: at 22.5 ppm, 29.5–30.0 ppm and 129.5–130.5 ppm. Combined with the marked reduction in the C-1 to C-4 resonances, these changes suggest there may have been some conversion of the [U-^{13}C]EPA to ^{13}C-enriched DPA (^{13}C enrichment at 29–30 ppm and 130 ppm) or DHA (^{13}C enrichment at 22.5 ppm). The loss of the C-1 to C-4 resonances is consistent with this possibility, but is also consistent with partial degradation of the [U-^{13}C]EPA to ^{13}C-enriched acetate from which saturated or monounsaturated fatty acids could be synthesized which also have signals at these chemical shifts. In contrast to the liver, ^{13}C signal intensities for fatty acid carbons of the carcass showed no consistent ^{13}C enrichment from the [U-^{13}C]EPA (Table 4).

DISCUSSION

Our aim in this study was to use high resolution ^{13}C NMR to monitor the presence of as many carbons of [U-^{13}C]EPA as possible in lipid extracts of liver and carcass so as to obtain information about possible oxidation compared with liver and carcass accumulation of EPA and its long chain metabolites, especially DHA. This is important because recent data suggest that >90% of dietary EPA is completely oxidized and therefore unavailable for metabolic or structural functions (13,14).

The high resolution ^{13}C NMR spectra showed that 24 h after injection, one or more ^{13}C-labelled n-3 fatty acids were present in the liver (Fig. 2). However, there was actually very low ^{13}C mass enrichment, and EPA-specific carboxyl terminal resonances virtually disappeared, which both agree with GLC data indicating minimal differences in EPA in the carcass or liver (Table 3). Thus, some of the weak ^{13}C enrichment may have been from DPA or DHA, *e.g.*, at C-3 of DHA (22.5 ppm), aliphatic carbons including C-5 of DPA (29–30 ppm) and at the "outer" olefinic carbons including C-7 of DPA (about 130 ppm). Some of the ^{13}C enrichment at 29–30 ppm and 130 ppm could also have arisen from a small amount of synthesis of monounsaturated fatty acids after degradation of [U-^{13}C]EPA. ^{13}C enrichment at these resonances was very weak, so basing net production of ^{13}C-enriched fatty acids on these data is speculative at this point. Nevertheless, high resolution ^{13}C NMR clearly has the potential to provide carbon-specific details of fatty acid synthesis and degradation.

Desaturation and elongation of EPA to DHA is dependent on the age of the animal and is influenced by fasting, anesthetic use, DHA and EPA level in the diet and the amount of substrate (EPA) given, so that the present data are subject to these constraints. When more [U-^{13}C]EPA or other [13C]-labelled fatty acids become available, several rats must be studied in each group so that more precise data about interanimal differences in metabolism of EPA and other fatty acids can be obtained. Nevertheless, we conclude that ^{13}C NMR may be able to assist in further investigation of these variables affecting metabolism and degradation of polyunsaturated fatty acids.

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Positional Distribution of n-3 Fatty Acids in Triacylglycerols from Rat Adipose Tissue During Fish Oil Feeding

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The present study was designed to investigate the metabolism of the n-3 polyunsaturated fatty acids (PUFA) in adipose tissue and its dependence upon dietary factors. Changes in the positional distribution of the fatty acids in triacylglycerols from retroperitoneal adipose tissue were studied as a function of time on rats fed for 4 wk a diet enriched with fish oil. The stereospecific analysis of triacylglycerols was based on random formation of *rac*-1,2-diacylglycerols by Grignard degradation. This was followed by synthesis of *rac*-phosphatidic acids and treatment with phospholipase A_2. In the triacylglycerols of the fish oil diet, 57% of the total n-3 fatty acids were in position 3, *i.e.*, two-thirds of 20:5n-3 and 22:5n-3 were esterified in *sn*-3 position, whereas 22:6n-3 was equally distributed in positions 2 and 3. After 4 wk of feeding fish oil, the fatty acid composition of adipose tissue triacylglycerols reached a steady state. Half of the n-3 fatty acids were found in position 3, namely 75% of 22:5n-3, 50% of 20:5n-3 and 18:4n-3 and 45% of 22:6n-3, the latter being equally distributed in positions 2 and 3. This pattern of distribution resembled that found in triacylglycerols of the fish oil diet, except for a higher proportion of 20:5n-3 in adipose tissue in position 1 at the expense of position 3. Throughout the 4-wk period of fish oil feeding, the distribution pattern of minor n-3 fatty acids (18:4n-3 and 22:5n-3) in adipose tissue triacylglycerols remained unchanged. On the other hand, at the onset of fish oil feeding, 20:5n-3 and 22:6n-3 became concentrated in position 3, but thereafter 20:5n-3 was progressively incorporated into position 1 and 22:6n-3 into position 2. We thus conclude that n-3 fatty acids are differentially esterified in triacylglycerols of white adipose tissue. Despite the complex sequence of hydrolysis and acylation steps involved, the positional distribution of n-3 fatty acids was found to be similar in both the fish oil diet and the stored fat, in contrast to what was observed for nonessential fatty acids.
Lipids 28, 279–284 (1993).

The beneficial effects of dietary intake of marine polyunsaturated fatty acids (PUFA), especially 20:5n-3 and 22:6n-3 (1), have attracted considerable interest during the past 15 yr. The results of animal and clinical studies have suggested that n-3 fatty acids may prevent or improve various pathological conditions, including atherosclerotic vascular degeneration (2), hyperlipidemia and hypercholesterolemia (3), insulin-dependent diabetes (4) and endotoxic shock (5), as well as cancer (6). Adipose tissue is the reservoir of fatty acids, both nonessential and essential, including n-3 PUFA. However, the influence of n-3 PUFA on the overall metabolism of adipose tissues is poorly understood. Moreover, the specific mechanisms controlling the storage and the mobilization of n-3 PUFA are unknown. The regiospecific synthesis of triacylglycerols depends on the selectivity of the transacylases involved in the glycerophosphate pathway (7), but the fatty acid specificity of these enzymes is not well understood. The regiospecificity of hormone-sensitive lipase from rat adipose tissue has been documented (8), but the effect of fatty acid chain length and unsaturation is not known (9). The positional specificity of this enzyme together with a nonrandom distribution of n-3 fatty acids in adipose triacylglycerols may account for the differential mobilization of these fatty acids that has recently been observed (Raclot, T., and Groscolas R., unpublished data). A better understanding of the metabolism of n-3 PUFA in adipose tissue requires knowledge of the positional distribution of n-3 PUFA in triacylglycerols. The effect of the positional distribution of fatty acids in dietary triacylglycerols on the time dependency of their distribution in adipose tissue is also not known. To gain insight into the metabolism of n-3 PUFA in adipose tissue, we have studied the specific distribution of n-3 PUFA incorporated into triacylglycerols. The triacylglycerols were purified from retroperitoneal adipose tissue in rats that were shifted from a basal diet to a fish oil diet for 1–4 wk. The data were compared with those obtained on the distribution of these fatty acids in the dietary triacylglycerols.

MATERIALS AND METHODS

Animals and diets. From weaning up to 8 wk of age, 12 male Wistar rats were individually housed in a room having a 12 h light/dark cycle at a constant temperature of 25°C. The animals were kept on a basal diet (A04, UAR, Villemoisson, France) containing 3% lipids. When the animals reached a 227 ± 8 g body weight, 9 rats were shifted to a semi-synthetic diet containing (g/kg): 450 sucrose, 250 caseine, 45 agar-agar, 45 minerals (mix 205B, UAR), 10 vitamins (mix 200, UAR), 10 sunflower oil, 190 purified fish oil (MaxEPA, R.P. Scherer, Beinheim, France). The amount of sunflower oil was chosen to meet the daily requirement in essential 18:2n-6. The fish oil diet was prepared weekly in bulk and supplemented with α-tocopherol (300 mg/kg) as an antioxidant. The diet was divided into daily rations and kept at −20°C. The fatty acid composition of the triacylglycerols of the diets is shown in Table 1. The phospholipid fraction present in the basal diet (17% of its total lipid moiety) was shown to have the same fatty acid composition as the triacylglycerol fraction.

Extraction and separation of lipids. Animals were killed by cervical dislocation at 9 a.m. Three rats fed the basal diet were killed at the onset of the experiment. Animals fed the fish oil diet were killed after 1 wk (280 ± 3 g), 2 wk (331 ± 22 g) or 4 wk (388 ± 26 g) of feeding (three rats at a time). These time periods were chosen based on results of preliminary studies which showed that the n-3 PUFA content in triacylglycerols of adipose tissue reached a steady-state level after 2 wk of feeding the fish oil enriched diet. Adipose tissue samples from retroperitoneal fat depots were immediately excised, and their total

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Abbreviations: DB, double bond index; EDTA, ethylenediaminetetraacetic acid; PUFA, polyunsaturated fatty acid(s); TLC, thin-layer chromatography; U/S, unsaturated/saturated fatty acid ratio.

lipids were extracted (10). Total lipids were similarly extracted from three pooled aliquots of the basal and the fish oil diets. The triacylglycerols were isolated by silicic acid column chromatography, using $CHCl_3$ for elution and were purified by thin-layer chromatography (TLC) on silica gel G (Merck, Darmstadt, Germany) using hexane/diethyl ether/acetic acid (70:30:1, by vol) as the developing solvent. Ethyl gallate was added to all solvents (25 mg/L) to minimize autoxidation, and reactions were carried out under nitrogen.

Generation of diacylglycerols. A procedure based on the Grignard degradation of about 10 mg of triacylglycerols was used as previously reported (11) without modification.

Preparation of phosphatidic acid. The first step of the diacylglycerol derivatization procedure (11) was performed with the following modifications. An ice-cooled solution of diacylglycerols (about 2 mg) in 0.2 mL of $CHCl_3$ was added slowly to 2 mL of a chilled phosphorus oxychloride solution ($CHCl_3$/pyridine/$POCl_3$; 9.5:9.5:1, by vol) with stirring, and the mixture was allowed to stand for 10 min at 0°C and for 50 min at 20°C. After the addition of 4 mL of $CHCl_3$, 2 mL of 0.5 M sodium hydrogen carbonate and 0.4 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA), the mixture was vortexed for 2 h and then centrifuged. The lower phase was evaporated, and the dried product dissolved in a mixture of $CHCl_3$/methanol (1:1, vol/vol) and partitioned with one volume of water. After evaporation of the solvent under nitrogen, the phosphatidic acid was purified by TLC (12).

Hydrolysis of phosphatidic acid. A procedure using snake venom phospholipase A_2 (13) was used with the following modifications. Phosphatidic acid (1–1.5 mg) was vortexed for 2 h at 25°C with 2 mL of diethyl ether and 1 mL of Tris buffer (50 mM Tris, 4 mM $CaCl_2$, pH 7.5) containing 5 units of *Crotalus atrox* phospholipase A_2 (Sigma, St. Louis, MO) dissolved in 20 μL buffer. The hydrolysis products were isolated by TLC (12). Released fatty acids (R_f = 0.83) would then reflect the fatty acid composition at the *sn*-2 position and lysophosphatidic acid (R_f = 0.49) is representative of the fatty acid composition at the *sn*-1 position of the triacylglycerols. The composition of the *sn*-3 position was calculated as 3 × (triacyl) − (*sn*-1 + *sn*-2 position).

The accuracy of the method was checked by the analysis of synthetic 1,3-dioleoyl-2-palmitoyl-*rac*-glycerol. A contamination not exceeding 2% (absolute) by fatty acids migrating from positions 1 and 3 to position 2 was observed, which is consistent with other procedures reported (13,14). Reproducible results were obtained by the method described although there remain large relative errors for minor components in position 3. Therefore, mole percentages lower than 0.5 mol% in positional distribution were omitted.

Fatty acids analysis. Fatty acids were converted to methyl esters by BF_3-catalyzed transmethylation (15) and analyzed by gas-liquid chromatography using a Chrompack CP9000 chromatograph equipped with a polar capillary column (AT-WAX, 60 m, i.d. 0.25 mm, Alltech). All solvents and chemicals were of reagent grade as supplied by Merck or Sigma. Results are given as means ± SEM. The Student's *t*-test was used to compare significant differences among means.

RESULTS

Dietary triacylglycerols. The composition and positional distribution of fatty acids in the triacylglycerols of the basal and fish oil diets are given in Table 1. For the basal diet, it was observed that the major fatty acid, 18:2n-6, showed no marked preference for any of the three glycerol positions. A similar trend was also observed in the fish oil diet. In this diet, most of the n-3 PUFA (18:4, 20:5 and 22:5) were preferentially esterified (to the extent of 65%

TABLE 1

Composition and Positional Distribution of Fatty Acids in Triacylgycerols from Basal and Fish Oil Diet[a]

Fatty acid	Basal diet position				Fish oil diet position			
	All	1	2	3	All	1	2	3
14:0	1.0	1.1	1.9	—[b]	8.6	9.3	16.5	—
16:0	16.9	24.3	11.5	14.1	21.1	25.4	33.5	4.3
18:0	2.0	4.0	1.7	—	3.7	6.2	2.0	2.8
18:1n-9	21.2	21.0	18.8	22.4	10.3	13.2	5.2	12.5
20:1n-9	1.1	1.1	1.0	1.3	1.5	1.7	0.7	2.0
16:1n-7	0.9	1.2	1.5	—	10.0	12.8	7.4	9.9
18:1n-7	1.5	2.9	1.1	—	3.6	5.9	1.7	3.1
18:2n-6	48.5	37.8	54.2	50.6	5.4	4.6	4.9	6.6
18:3n-3	3.8	3.0	3.4	4.5	0.7	0.8	—	0.8
18:4n-3	0.2	—	—	—	3.6	3.9	1.3	5.6
20:4n-3	0.0	—	—	—	0.9	1.0	—	1.4
20:5n-3	0.8	—	0.6	1.4	15.9	8.7	7.8	31.0
22:5n-3	0.0	—	—	—	1.6	0.6	1.0	3.3
22:6n-3	1.0	—	0.7	1.9	9.4	2.0	13.4	12.8
Sum n-3	5.8	3.0	4.7	7.8	32.1	17.0	23.5	54.9

[a]Mol % in each position. Values are from one representative analysis of a pool of three samples of each diet. Columns do not total 100% as fatty acids contributing less than 1 mol% in the three positions were not included. "All" includes fatty acid composition of all the triacylglycerols of the diet.
[b]Less than 0.5 mol% for the positional distribution data.

TABLE 2

Fatty Acid Composition of Triacylglycerols in Retroperitoneal Adipose Tissue from Rats Fed Basal and Fish Oil Diets[a]

Fatty acid	Basal diet	Fish oil diet		
		1 wk	2 wk	4 wk
14:0	1.9 ± 0.1	3.8 ± 0.2	4.9 ± 0.1	5.6 ± 0.2
16:0	28.9 ± 0.2	31.7 ± 0.7	32.8 ± 0.4	30.0 ± 0.3
18:0	3.6 ± 0.1	3.5 ± 0.1	4.1 ± 0.2	4.6 ± 0.1
18:1n-9	25.4 ± 0.4	21.6 ± 0.5	18.8 ± 0.4	18.8 ± 0.6
16:1n-7	7.2 ± 0.1	9.0 ± 0.4	9.0 ± 0.2	8.7 ± 0.1
18:1n-7	3.0 ± 0.1	3.2 ± 0.1	3.3 ± 0.2	4.1 ± 0.1
18:2n-6	24.9 ± 0.7	16.4 ± 0.9	11.5 ± 0.1	11.1 ± 0.4
18:3n-3	1.5 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
18:4n-3	0.1 ± 0.0	0.7 ± 0.1	1.2 ± 0.1	1.5 ± 0.1
20:4n-3	–[b]	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
20:5n-3	0.2 ± 0.0	2.8 ± 0.3	4.5 ± 0.2	4.4 ± 0.5
22:5n-3	0.4 ± 0.1	1.1 ± 0.2	1.4 ± 0.4	1.4 ± 0.1
22:6n-3	0.6 ± 0.1	2.5 ± 0.3	3.9 ± 0.1	4.3 ± 0.2
Sum n-3	2.8 ± 0.1	8.3 ± 0.8	12.3 ± 0.3	13.0 ± 0.9

[a]Mol % of total fatty acids. Mean ± SEM of three experimental values.
[b]Less than 0.1 mol %.

for 20:5) in position 3. On the other hand, 22:6n-3 was bound almost exclusively in positions 2 and 3. Despite the low amount of n-3 PUFA in the basal diet, a trend toward preferential esterification of 20:5 and 22:6 in position 3 was observed.

Adipose tissue triacylglycerols. Feeding of the fish oil diet rapidly increased the concentration of the n-3 fatty acids in white adipose tissue (Table 2), except for 18:3n-3 whose concentration was significantly ($P < 0.02$) lowered after one week of feeding. The broad fatty acid composition of the adipose tissue triacylglycerols resembled that of the dietary triacylglycerols. Thus, after 4 wk, the high contents of the n-3 PUFA found typically in the fish oil diet were also observed in adipose tissue triacylglycerols. Similarly, the diet and the fat store shared low contents in n-9 monounsaturated fatty acids and in n-6 PUFA. The positional distribution of the main fatty acids in the triacylglycerols from the rat adipose tissues is given in Table 3. In rats fed the basal diet, each position of the triacylglycerols showed a discrete fatty acid pattern. Fatty acids at position 1 were highly saturated (unsaturated/saturated ratio, U/S = 0.8), about half of the 16:0 and 18:0 contents being found in this position. Fatty acids at position 3 were significantly ($P < 0.001$) more unsaturated (U/S = 2.4) than those at position 1; monoenoic acids (mainly 18:1n-9) and n-3 PUFA were preferentially esterified in this position. The highest degree of unsaturation (U/S = 4.6) was found at position 2, owing to a high concentration of 18:2n-6. A similar pattern was observed for the double bond index (DB), for which the order of increasing values corresponded to positions 1, 3 and 2, respectively. In rats fed the fish oil diet during 4 wk and having reached a steady-state fatty acid composition, the positional distribution of the saturated fatty acids remained closely similar to that observed in rats fed the basal diet. Of the monoenoic fatty acids, only 18:1n-9 was significantly affected; the amount of this acid at position 3 had decreased by one-half after 4 wk ($P < 0.01$ *vs.* basal diet). Although the 18:2n-6 content decreased more than twofold ($P < 0.001$ *vs.* basal diet) in triacylglycerols, the distribution pattern of this fatty acid remained unchanged with a high concentration in position 2 (between 50 and 70% of the total amount). Thus, despite the large increase in n-3 PUFA after 4 wk of feeding with the fish oil diet, the distribution of the other fatty acids at positions 1, 2 and 3 of glycerol was not markedly modified. The greatest extent of saturation was found at position 1 and the lowest at position 2. The main n-3 PUFA in adipose tissue triacylglycerols showed three different distribution patterns (Table 3, Fig. 1): (i) 22:5 was mainly concentrated at position 3 (75%), the remaining part being equally distributed between positions 1 and 2; (ii) 18:4 and 20:5 were found mainly at position 3 (50%), only 15% being found at position 2; (iii) 22:6 was equally distributed among positions 2 and 3 after 2 wk, with only 15% being found at position 1. In summary, when a steady fatty acid composition was reached, half of the total n-3 PUFA was found at position 3, the remaining part being equally distributed between positions 1 and 2. However, the proportion of each n-3 PUFA at position 3 decreased in the sequence 22:5 > 20:5 = 18:4 > 22:6 > 18:3, with only 18:3 and 22:6 showing a significant accumulation at position 2. From Table 4, it is evident that there is a close resemblance between triacylglycerols of the adipose tissue and those of the diet. Nevertheless, a higher esterification of 20:5 at position 1 was noted in adipose tissue, at the expense of position 3. It was observed that the time-dependent distribution of the minor n-3 PUFA of the fish oil diet (18:4 and 22:5) remained unchanged during the feeding period (Fig. 1). In contrast, the patterns displayed by the major n-3 PUFA (20:5 and 22:6) (Fig. 1) changed in the course of feeding from 1 to 4 wk. At the onset of feeding with fish oil, 20:5 and 22:6 accumulated preferentially in position 3. The final level of these n-3 fatty acids in position 3 was already reached one week after the diet was changed, thus leading to an increase in DB in this position reaching its highest value. Later, after 2 and 4 wk of feeding, 20:5 and 22:6 were progressively incorporated into positions 1 and 2, respectively.

DISCUSSION

Positional distribution of n-3 PUFA in adipose tissue triacylglycerols as a function of their distribution in the diet.

TABLE 3

Positional Distribution of Fatty Acids in Triacylglycerols of Retroperitoneal Adipose Tissue from Rats Shifted from a Basal Diet to a Fish Oil Diet[a]

Fatty acid	Basal diet position			1 wk fish oil diet position			2 wk fish oil diet position			4 wk fish oil diet position		
	1	2	3	1	2	3	1	2	3	1	2	3
14:0	1.9 ± 0.4	1.3 ± 0.4	2.6 ± 0.7	4.0 ± 0.4	3.1 ± 0.5	4.0 ± 0.8	5.2 ± 0.2	5.4 ± 0.3	4.1 ± 0.1	5.1 ± 1.1	4.2 ± 2.3	7.7 ± 2.4
16:0	49.6 ± 1.1	14.3 ± 0.5	22.8 ± 0.8	52.1 ± 0.8	17.6 ± 0.6	24.1 ± 1.7	51.8 ± 0.6	23.4 ± 1.0	23.3 ± 0.8	43.2 ± 2.2	20.5 ± 2.2	26.3 ± 3.8
18:0	4.8 ± 0.8	2.1 ± 0.3	3.8 ± 0.8	6.1 ± 0.9	3.4 ± 0.8	1.0 ± 0.5	5.2 ± 0.2	2.4 ± 0.4	—	7.5 ± 1.8	3.0 ± 0.8	3.2 ± 2.1
18:1n-9	16.3 ± 0.4	26.6 ± 0.9	33.2 ± 0.1	12.8 ± 0.6	28.8 ± 1.4	21.9 ± 2.1	9.9 ± 0.4	22.6 ± 1.2	23.8 ± 0.2	12.8 ± 1.5	25.8 ± 3.4	17.8 ± 2.5
16:1n-7	6.7 ± 0.3	6.3 ± 0.4	8.5 ± 1.0	6.6 ± 0.2	11.8 ± 0.8	8.0 ± 1.2	6.8 ± 0.2	10.9 ± 0.5	9.2 ± 0.2	6.4 ± 1.1	9.2 ± 1.6	10.7 ± 1.8
18:1n-7	4.3 ± 0.2	1.7 ± 0.2	3.0 ± 0.2	4.5 ± 0.1	2.4 ± 0.3	2.7 ± 0.1	4.7 ± 0.2	2.6 ± 0.0	2.5 ± 0.3	6.7 ± 0.5	2.8 ± 0.3	2.8 ± 0.5
18:2n-6	13.1 ± 0.8	43.8 ± 1.2	17.8 ± 0.6	6.8 ± 0.2	23.5 ± 1.7	17.9 ± 1.6	5.3 ± 0.2	21.4 ± 1.0	7.8 ± 0.3	5.6 ± 0.5	22.5 ± 2.6	5.1 ± 1.1
18:3n-3	1.4 ± 0.1	1.9 ± 0.1	1.2 ± 0.1	0.6 ± 0.2	1.3 ± 0.2	1.0 ± 0.3	0.6 ± 0.0	1.2 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	1.2 ± 0.0	0.6 ± 0.1
18:4n-3	—[b]	—	—	0.5 ± 0.1	0.5 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	0.8 ± 0.0	2.1 ± 0.3	1.2 ± 0.2	0.8 ± 0.2	2.6 ± 0.3
20:4n-3	—	—	—	—	—	—	0.7 ± 0.1	—	0.7 ± 0.0	1.0 ± 0.1	—	0.7 ± 0.2
20:5n-3	—	—	—	1.0 ± 0.2	0.6 ± 0.1	6.3 ± 0.6	4.1 ± 0.4	2.2 ± 0.1	7.4 ± 0.2	4.5 ± 0.3	1.9 ± 0.5	7.0 ± 1.2
22:5n-3	—	—	1.0 ± 0.2	—	—	2.5 ± 0.7	0.5 ± 0.1	0.5 ± 0.1	3.3 ± 1.2	0.5 ± 0.0	0.6 ± 0.1	3.2 ± 0.4
22:6n-3	—	0.7 ± 0.3	1.1 ± 0.3	0.7 ± 0.3	1.3 ± 0.1	5.3 ± 1.0	1.7 ± 0.1	4.6 ± 0.2	5.5 ± 0.0	2.0 ± 0.2	5.2 ± 0.4	5.9 ± 0.5
U/S	0.8 ± 0.1	4.6 ± 0.1	2.4 ± 0.1	0.6 ± 0.0	3.2 ± 0.2	2.4 ± 0.5	0.6 ± 0.0	2.2 ± 0.1	2.1 ± 0.1	0.8 ± 0.1	2.7 ± 0.3	1.7 ± 0.4
DB	61.0 ± 4.0	135.0 ± 2.0	105.0 ± 4.0	60.0 ± 8.0	125.0 ± 9.0	170.0 ± 9.0	77.0 ± 1.0	132.0 ± 0.0	160.0 ± 5.0	88.0 ± 4.0	139.0 ± 4.0	155.0 ± 9.0

[a]Fatty acid composition in mol% in each position. Mean ± SEM of three experimental values.
[b]Less than 0.5 mol%. See legend of Table 1 for other explanations. U/S, unsaturated/saturated fatty acid ratio; DB, double bond index (number of double bonds per 100 mol fatty acids).

TABLE 4

Positional Distribution of Major n-3 Polyunsaturated Fatty Acids in Triacylglycerols from Fish Oil Diet (D) and White Adipose Tissue (WAT) of Rats Fed This Diet for Four Weeks[a]

Fatty acid	Position 1		Position 2		Position 3	
	D	WAT	D	WAT	D	WAT
20:5n-3	18	33	16	14	66	53
22:6n-3	7	15	48	40	45	45
18:4n-3	36	26	12	17	52	57
22:5n-3	12	11	20	14	68	75

[a]Mol% for each fatty acid, the sum of the three positions being 100. Fatty acids are arranged according to their decreasing content in the fish oil diet.

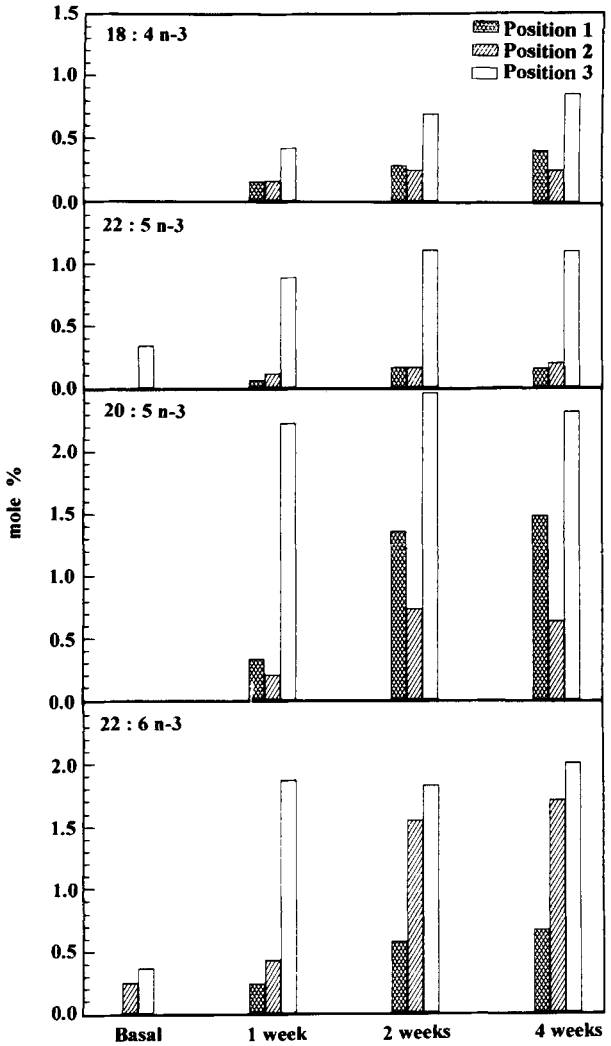

FIG. 1. Positional distribution as a function of time of n-3 fatty acids in retroperitoneal adipose tissue triacylglycerols from rats fed a fish oil diet. For each fatty acid, the sum of the three columns at each time represents its total amount (in mol%) in triacylglycerols (data recalculated from Table 3).

The incorporation of fatty acids into triacylglycerol stores is still poorly understood. Several factors may be involved in the control of the positional distribution of n-3 fatty acids in adipose tissue triacylglycerols, including their

immediate availability through intestinal absorption (16) and the specificity of acyltransferases (17) as well as of lipolytic enzymes (9,18). The structure of dietary triacylglycerols should also be taken into account. Recent studies (19,20) have demonstrated that fatty acids located at position 2 in dietary fish oil tend to remain in this position in circulating triacylglycerols. This is unexpected in view of the hydrolytic action of pancreatic lipase in the intestinal lumen and the complex processing of absorbed lipids in mucosal cells. The finding of a selective inhibitory action of 20:5n-3 on the rate of hydrolysis of triacylglycerols in fish oil chylomicron by lipoprotein lipase (21) suggests that fatty acids may be selectively directed toward the peripheral tissues. These observations may have important implications for the incorporation of n-3 fatty acids into adipose tissue stores. Several reports have been published on the positional distribution of fatty acids in fish oils (16,18,22–25). Only two of these studies were conducted with the Grignard reagent (16,22), which eliminates biases due to the well-known fatty acid specificity of pancreatic lipase (18). In 1963, a typical fish pattern with accumulation of polyenoic acids at position 2 of triacylglycerols was described (23), but this pattern would appear to be provisional rather than firmly established. The distribution of the two main n-3 fatty acids presented here for MaxEPA oil (20:5 mainly in position 3 and 22:6 in positions 2 and 3) is in marked contrast to that reported for menhaden oil (22) (20:5 mainly in positions 2 and 3, whereas 22:6 is principally located in position 2). This distribution is also different from that reported for a commercial preparation consisting largely of sardine oil (16) (20:5 evenly distributed among all three positions).

Previous studies on the distribution of n-3 fatty acids in triacylglycerol stores (23–27) have not involved defined diets. Hence, no information is available on the relationship between the structures of dietary n-3 enriched oils and those of the stored triacylglycerols. Animals fed the basal diet in the present study had adipose tissue triacylglycerols with the essential fatty acid 18:2n-6 mainly in position 2. The nonessential fatty acids of exogenous and endogenous origin were equally distributed between the two other positions, with 16:0 and 18:1n-9 being found mainly in positions 1 and 3, respectively. This distribution is similar to that previously reported for the rat and other mammals (25,28,29). After feeding fish oil, the triacylglycerol composition was profoundly modified, leading to a decrease in the 18:2n-6 content with compensation by an increase in the n-3 fatty acids. The distribution pattern of the n-3 fatty acids rapidly changed and thereafter remained stationary after a feeding period of two weeks. With respect to 20:5, 22:5 and 22:6, the resulting pattern was different from that reported in rat and mink fed with a mackerel diet (25) and from the pattern observed for wild polar bear and seal (25). No conclusion can be drawn from these differences since no information on the molecular structure of the dietary triacylglycerols was obtained in these studies. Our observation of a close resemblance between the position of n-3 fatty acids in triacylglycerols of the fish oil diet and those of the rat adipose tissue suggests that certain biosynthetic pathways in fish and rat may be similar. Unfortunately, no further light has been shed on this question by studies on mammals fed various marine oils (23,24,26). However, rat enterocytes purportedly transfer triacylglycerols to the lymph with a positional distribution of n-3 fatty acids similar to that of the consumed fish oil (20). Further studies of the metabolic chain following feeding with fish oil or fatty acid ethyl esters (19) are clearly necessary.

Incorporation of n-3 PUFA into adipose tissue triacylglycerols as a function of specific mechanisms. The rapid incorporation of the main n-3 fatty acids (20:5 and 22:6) observed in position 3 or adipose tissue triacylglycerols after feeding rats with the fish oil diet suggests that the enzyme diacylglycerol acyltransferase has a high selectivity for these fatty acids. This enzyme is known to play a key role in the final step of triacylglycerol synthesis (17). However, specific acylation in this position under our experimental conditions may also reflect in part the composition of the changing adipocyte acyl-CoA pool. The relative importance of enzyme properties *vs.* substrate availability remains to be determined. The observation of the delayed and concomitant channelling of 20:5 and 22:6 into positions 1 and 2 indicates that the two acylating enzymes along the glycerol-3-phosphate pathway have a much lower affinity for n-3 than for nonessential fatty acids. Nevertheless, our results suggest some specificity of the 1- and 2-acylglycerophosphate acyltransferases for 20:5 and 22:6, respectively. As was previously suggested for adipose tissue (7,17,25,30) and liver (31,32), the relative affinity and thus the competition of different polyunsaturated fatty acids for acylation into triacylglycerols may be an important factor for the asymmetric distribution. As for saturated fatty acids in rats fed coconut oil (33), it can be postulated that the delayed changes in 20:5 and 22:6 at positions 1 and 2 may also arise from an intermolecular rearrangement of triacylglycerol fatty acids. The extent of this intracellular remodelling is not yet certain, but the present results may be of help in designing future experiments on n-3 PUFA recycling in the pool of adipocyte triacylglycerols. The existence of a monoacylglycerol pathway in adipose tissue (34) and the recent demonstration of a partial retention of fish oil structure in chylomicron triacylglycerols (19) illustrate the diversity of mechanisms involved in depot fat remodelling.

Since interconversions of triacylglycerols and phospholipids through diacylglycerols are well documented, adipocyte membrane phospholipids are likely to contribute to the formation of diacylglycerols. These intermediates may be secondarily acylated to form highly unsaturated triacylglycerol species. The rapid incorporation of dietary n-3 PUFA into adipose tissue phospholipids (Leray *et al.*, unpublished data) is consistent with a contribution of membrane phospholipids in adipose triacylglycerol biosynthesis. From our observations and the reported similarity between the structures of the 1,2-diacylglycerol moiety in rat liver triacylglycerols and in phosphatidylcholine (32), one still needs to answer the question to what extent adipose lipid stores are influenced by cellular phospholipids. In conclusion, the present study has shown that considerable amounts of n-3 PUFA can be stored in rat adipose tissue and that these fatty acids are differentially esterified in triacylglycerols. Since the positional distribution of n-3 PUFA in the fish oil ingested is similar to that of the lipid stores, further information on their relationships would be desirable. It could be obtained through studies with natural and randomized fish oil or n-3 PUFA ethyl esters as the diet.

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Relationship Between Mouse Liver Δ9 Desaturase Activity and Plasma Lipids

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This study was undertaken to investigate the total plasma fatty acid composition and the relationship between plasma triacylglycerol (TG) levels and liver Δ9 desaturase activity in mice fed n-3 and/or n-6 fatty acid or hydrogenated coconut oil (HCO) (maximum 25 mg/g) supplemented diets. Generally, plasma TG levels and Δ9 desaturase activity were inversely correlated with the ratio of the sum of long chain n-6 fatty acids to 18:2n-6 and to the ratio of the sum of long chain n-3 fatty acids to 18:n-3, but they were positively correlated with the ratio of products and substrates (18:1/18:0) of the enzyme in plasma total lipids. The n-3 fatty acid (mainly 20:5n-3) enriched diet, when compared to the HCO diet at 21 d, caused a significant reduction in plasma TG levels but not in Δ9 desaturase activity. However, a marked reduction in plasma TG content (50–60%) and Δ9 desaturase activity (55–70%) was observed when both 20:5n-3 and 18:3n-6 were supplemented in the diet. The plasma TG levels and Δ9 desaturase activity rose again when the animals were fed the HCO diet or chow. The results suggest that low dose supplementation of a mixture of n-3 (mainly 20:5n-3) and n-6 (18:3n-6) fatty acids modified both plasma TG content and liver Δ9 desaturase activity, in parallel.
Lipids 28, 285–290 (1993).

Both n-3 and n-6 polyunsaturated fatty acids modulate plasma lipid composition and hepatic lipid metabolism. There also is considerable evidence that the n-3 fatty acids, present in fish oil, can reduce plasma triacylglycerol (TG) levels in normal and hyperlipidemic subjects (1,2) even with high linoleic acid intake (3). This effect has been attributed to the fact that n-3 fatty acids may inhibit very low density lipoprotein (VLDL) TG synthesis (2,4), promote the clearance of VLDL from the circulation (5) and reduce hepatic lipogenic enzyme activity (6). Moreover, it has been reported that n-3 fatty acids suppress fatty acid synthesis in isolated rat hepatocytes (7) and reduce Δ9 desaturase activity *in vivo* (8,9). In contrast, polyunsaturated fatty acids of the n-6 series, such as linoleic acid (18:2n-6) or its desaturation product γ-linolenic acid (18:3n-6), do no reduce plasma TG levels either in healthy or hypertriglyceridemic patients (3,10,11).

We have shown in a previous study that diets supplemented with 18:3n-6 together with n-3 fatty acids (mainly 20:5n-3) increase the levels of these fatty acids and their metabolites in liver microsomes (12). We have suggested that the increase in long chain n-3 and n-6 fatty acid metabolites might be responsible for the inhibition of microsomal Δ9 desaturase activity (12). However, the combined effect of a mixture of these n-3 and n-6 free fatty acids on plasma TG levels was not examined. The present paper describes the relationships between plasma TG levels and liver Δ9 desaturase activity and between plasma fatty acid composition and desaturase activity in mice fed n-3 and n-6 fatty acid supplemented diets.

MATERIALS AND METHODS

Chemicals. All organic solvents and chemicals were of reagent grade and obtained from British Drug House Inc. (Toronto, Ontario, Canada). Fatty acid standards were purchased from Nu-Chek-Prep (Elysian, MN). [1-^{14}C]Palmitic acid (more than 98% radiochemical purity; 9 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and used without further purification. γ-Linolenic acid concentrate and fish oil concentrate were obtained from Callanish Ltd. (Isle of Lewis, Scotland). The free n-3 and n-6 fatty acids were prepared by urea inclusion, saponification and further purification by silicic acid chromatography following procedures described elsewhere (13).

Animals and diets. Female FVB mice (28-days-old) were obtained from Charles River Canada Breeding Laboratories (Montreal, Quebec, Canada). Animals were housed three per cage in a temperature-controlled environment (22 ± 2°C) with a 12 h light/dark cycle. All mice were adapted for 1 wk to Rodent Laboratory Chow No. 5001 diet (Ralston Purina Co., St. Louis, MO) and water *ad libitum*. Mice were then weighed and randomly allocated to one of the experimental groups. All diets were prepared using Rodent Laboratory Chow No. 5001 supplemented with 25 mg/g hydrogenated coconut oil (HCO) (Teklad Test Diets, Madison, WI) or replaced with mixtures of n-6 and n-3 free fatty acid concentrates. When diets were supplemented with 18:3n-6 and 20:5n-3 alone or in a mixture, these fatty acids were maintained at comparable levels. The saturated and other unsaturated fatty acids were also kept at similar levels. The final fatty acid composition of each of these diets is shown in Table 1.

Three different experimental feeding schedules were used. In Experiment 1, mice were fed a diet rich in both eicosapentaenoic acid (EPA) and γ-linolenic acid (GLA) (diet EPA/GLA), rich in EPA only (diet EPA), rich in GLA only (diet GLA) or supplemented with HCO in place of both n-3 and n-6 fatty acids (diet HCO) for 21 d. In Experiment 2, mice were fed either diet EPA/GLA or HCO for 7 d and then crossed over onto the other diet for 14 d. In Experiment 3, mice were fed diets EPA/GLA, EPA, GLA, or HCO for 7 d and then switched back to chow for a washout period of 14 d. All diets were fed *ad libitum*. Fresh diets were prepared every second day with free fatty acids that were kept under N_2 at −20°C.

Every 7 d, at least three mice from each experiment were put under ether anesthesia, and blood was drawn by cardiac puncture into heparinized syringes before sacrifice.

Isolation of hepatic microsomes. Nonfasted mice were sacrified at 9 a.m. to avoid circadian variations in enzyme activity (14) and to obtain the maximum activities of liver desaturases (14,15). Microsomes were isolated by differential ultracentrifugation as previously described (16). The

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Abbreviations: EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; GLA, γ-linolenic acid; HCO, hydrogenated coconut oil; HPLC, high-performance liquid chromatography; P/S, polyunsaturated to saturated fatty acid ratio; PUFA, polyunsaturated fatty acids; TG, triacylglycerols; VLDL, very low density lipoprotein.

TABLE 1

Fatty Acid Composition of Diets[a]

Fatty acid	Diets				
	EPA/GLA	EPA	GLA	HCO	CHOW
12:0	trace	trace	trace	19.0	trace
14:0	1.3	1.3	1.2	7.2	1.3
16:0	9.2	9.2	9.2	10.1	9.3
16:1	4.3	5.2	1.8	1.8	1.9
18:0	2.8	3.0	2.8	3.7	2.8
18:1	11.5	13.6	11.7	11.6	11.7
18:2n-6	15.4	16.0	15.9	16.4	15.1
18:3n-6	10.3	0.2	14.3	0.1	trace
18:3n-3	2.2	2.1	2.1	2.0	2.2
18:4n-3	1.5	1.6	0.4	0.4	0.4
20:4n-6	0.4	0.4	0.2	0.2	0.2
20:5n-3	12.1	13.9	1.2	1.2	1.2
22:4n-6	0.6	0.8	0.3	0.3	0.3
22:5n-3	0.9	1.0	0.9	0.1	0.1
22:6n-3	2.6	3.4	1.3	1.3	1.3
Total	75.1	71.7	63.3	75.4	47.8

[a]Values shown are in mg/g diet calculated from gas chromatographic analysis. EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; HCO, hydrogenated coconut oil; CHOW, Purina Rodent Chow, No. 500.

microsomal pellet was suspended in 0.25 M sucrose and 62 mM phosphate buffer solution and kept frozen at −80°C until used. The protein concentration was measured by the method of Lowry *et al.* (17) with bovine serum albumin as standard.

Desaturase assays. The activity of Δ9 desaturase was determined by measuring the conversion of [1-^{14}C]16:0 (palmitic acid) to [1-^{14}C] 16:1n-7 (palmitoleic acid). Reactions were started by adding microsomal protein (5 mg) to pre-incubated tubes containing 0.25 μCi of 16:0 (final concentration 53.3 μM) and ATP (1.30 mM), NADH (0.87 mM) and coenzyme A (0.06 mM) as described (18). After 15 min incubation in a shaking water bath (37°C), the reactions were stopped with 2 mL 10% (wt/vol) KOH in ethanol. Lipids in the incubation mixture were saponified at 80°C for 45 min under N_2. After acidification, fatty acids were extracted with hexane and then methylated with BF_3 in methanol at 90°C for 30 min (19).

Lipid analysis. Lipids were extracted from plasma with chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (20). The composition of fatty acid methyl esters (FAME) in plasma lipids was determined by gas-liquid chromatography as described previously (21). Radiolabelled FAME from the desaturase studies were analyzed by high-performance liquid chromatography (HPLC) essentially according to the method of Narce *et al.* (22). The HPLC equipment (Waters Associates, Milford, MA) included a variable wavelength UV-vis monitor (set at 205 nm), a radioisotope detector (model 171, Beckman, Fullerton, CA, with 96% efficiency for ^{14}C detection) and an ultrasphere ODS column 25 cm × 4.6 mm i.d. (5 μ particle size, Beckman). FAME were separated isocratically with acetonitrile/water (95:5, vol/vol) at a flow rate of 1 mL/min and were identified by comparison with authentic standards.

Plasma TGs were measured enzymatically on a Cobas-Bioanalyzer (Roche Analytical Instruments Inc., Nutley, NJ) using the La Roche MA-Kit.

Statistical analysis. The results are expressed as means ± SD. Significance was assessed by one-way analysis of variance and multiple range tests using the SPSS-PC+ software package (SPSS Inc., Chicago, IL). Regression analysis of desaturase activity and plasma TG content or fatty acid ratios was made using the least-square method.

RESULTS

Table 2 shows that mouse body weight, the liver weight/body weight × 100 ratio and food consumption were not affected by n-6 or n-3 long chain fatty acid enriched diets or the HCO diet at the end of the 3-week feeding period. These parameters were also not significantly different among different dietary groups at any other time of sacrifice. This indicated that the amounts and ratios of n-3 and n-6 fatty acids added to the diets were acceptable.

Figure 1, panel A shows the time-dependent changes in plasma TG from mice initially fed the HCO diet for 7 d and then changed to the EPA/GLA diet or to chow for a period of 14 d. Panel B shows the plasma TG levels in animals fed the EPA/GLA diet for either 21 d or for 7 d and then changed to either chow or HCO diet for 14 more days. The plasma TG content in all animals fed the EPA/GLA diet was reduced by 50-60% (Fig. 1, panels A and B). Replacing the EPA/GLA diet at day 7 with the HCO diet or chow for 14 more days led to a significant increase (maximum 81% from day 7 values) in plasma TG levels (Fig. 1, panel B). No significant changes in the plasma TG levels were observed in animals initially fed either the EPA or GLA diet or when replaced with chow (Fig. 1, panels C and D, respectively).

When the 18:1/18:0 ratios of plasma total lipids from all animals fed different diets were plotted against plasma TG content, a positive relationship was observed (Fig. 2, panel A). In contrast, plasma TG levels were negatively

TABLE 2

Effect of Different Diets on Food Intake, Body Weight and Liver Weight[a]

Diet	Food intake (g/d)	Body weight[b] (g)	Liver weight/body weight[b] × 100
HCO	3.70 ± 0.49	23.66 ± 2.08	6.50 ± 0.60
EPA/GLA	2.89 ± 0.33	20.80 ± 3.36	6.10 ± 0.60
EPA	3.12 ± 0.35	20.86 ± 2.34	6.30 ± 0.30
GLA	3.31 ± 0.20	21.37 ± 2.07	6.10 ± 0.40
Chow	3.67 ± 0.46	21.41 ± 1.41	6.00 ± 0.80

[a]Each value represents the mean ± SD from at least three mice fed the diets for 21 d. Differences were not significant among all diets. For abbreviations, see Table 1.
[b]At time zero, body weight: 19.66 ± 2.13 g, liver weight/body weight × 100: 6.10 ± 0.60.

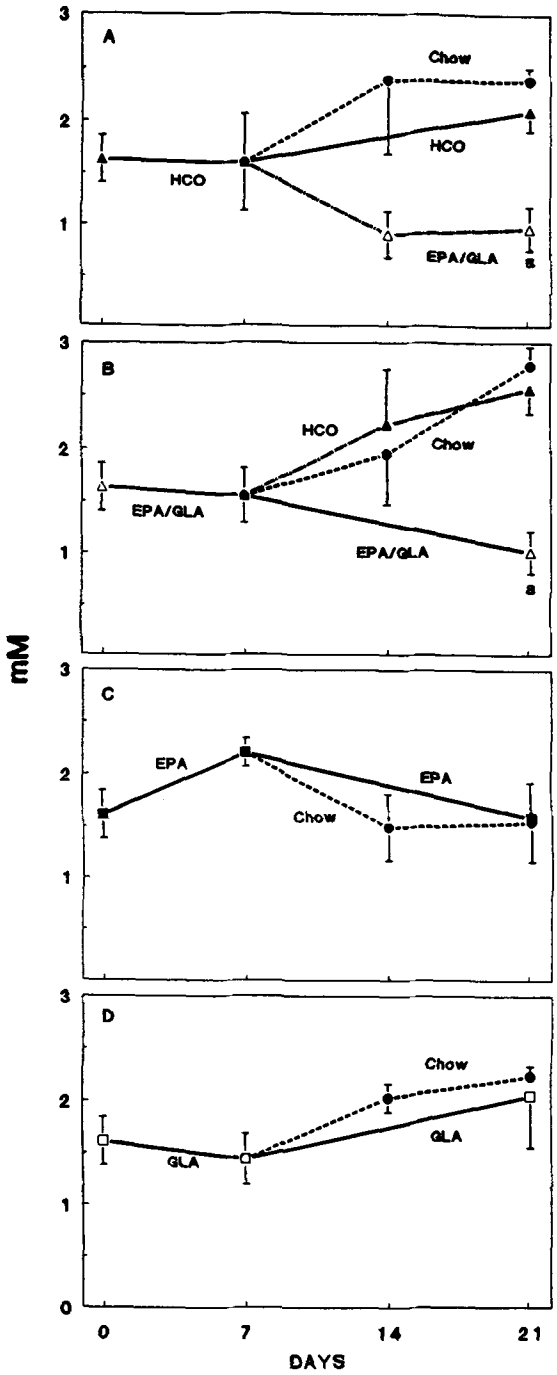

FIG. 1. Plasma triacylglycerol levels of mice fed n-3 and n-6 polyunsaturated fatty acid supplemented diets. Mice were fed one of the following diets as described in Material and Methods: ▲, hydrogenated coconut oil (HCO); △, eicosapentaenoic acid/γ-linolenic acid (EPA/GLA); ■, EPA; □, GLA; ●, Chow. Diets fed continuously throughout the experiment are denoted by solid lines. Diets changed on day 7 are indicated by dotted lines. Each value represents the mean ± SD of at least three mice. [a]Significantly different from 7-day values (panel A) or 0-day values (panel B) at $P < 0.01$.

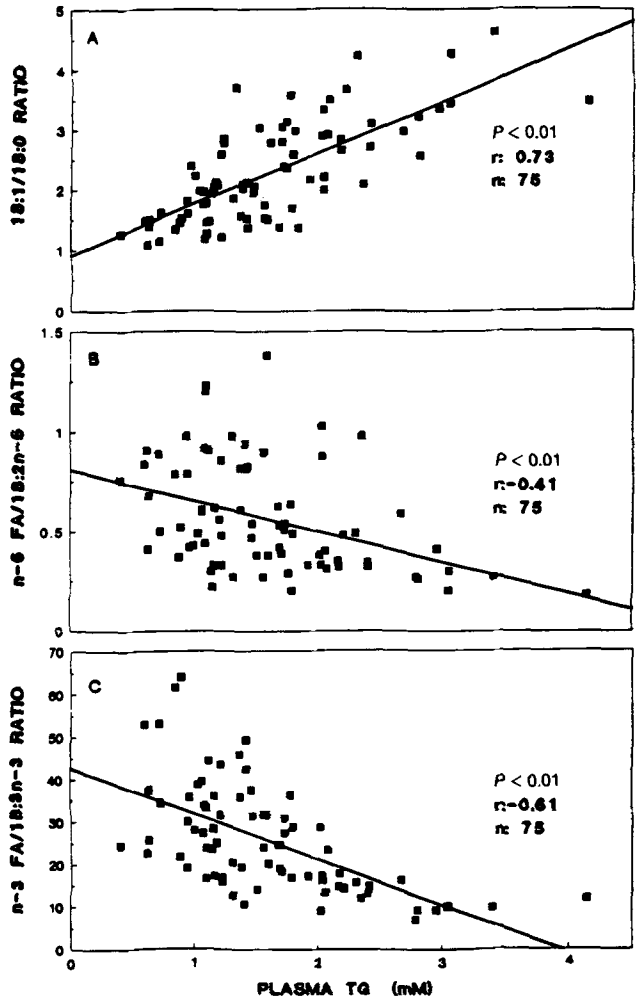

FIG. 2. Relationships between plasma fatty acids (FA) and triacylglycerols (TG). Correlations were calculated by linear regression analysis using data obtained from mice in all dietary groups at all times of sacrifice (n = 75). Total n-6 and n-3 fatty acids were related to their corresponding precursors 18:2n-6 and 18:3n-3, respectively, in order to normalize the differences found in plasma 18:2n-6 and 18:3n-3 content.

correlated to the ratio of the sum of long chain n-6 fatty acids/18:2n-6 or the ratio of the sum of long chain n-3 fatty acids/18:3n-3 (Fig. 2, panels B and C, respectively).

Liver Δ9 desaturase activity was readily affected by dietary modification. Feeding mice with the EPA/GLA diet for 7 to 21 d reduced Δ9 desaturase activity by 55 to 70% (Fig. 3, panels A and B). In mice previously fed the EPA/GLA diet, HCO feeding gradually increased the Δ9 desaturase activity; however, feeding chow for 14 d returned the Δ9 desaturase activity to day zero values. Feeding the HCO, EPA or GLA enriched diets throughout had no significant effect on Δ9 desaturase activity (Fig. 3, panels A, C and D, respectively).

Correlations between liver Δ9 desaturase activity and different fatty acids of plasma total lipids were also analyzed. Enzyme activity was positively correlated with the ratio of 18:1/18:0 (Fig. 4, panel A) whereas it was negatively correlated with the ratio of the sum of long chain n-6 fatty acids to 18:2n-6 or the ratio of the sum of long chain n-3 fatty acids to 18:3n-3 (Fig. 4, panels B and C, respectively).

DISCUSSION

Lawson and Hughes (23) have previously shown that free fatty acids were more efficiently absorbed than their re-

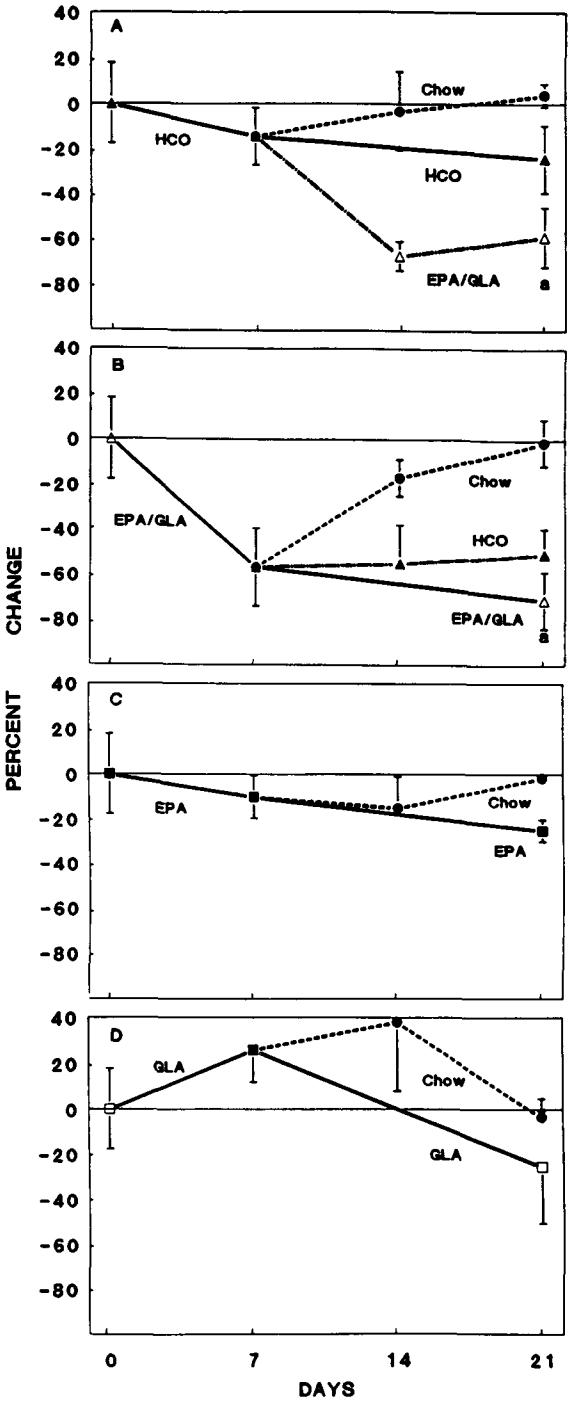

FIG. 3. Percent change of Δ9 desaturase activity of mice fed n-3 and n-6 polyunsaturated fatty acid supplemented diets. Mice were fed one of the following diets as described in Materials and Methods: ▲, hydrogenated coconut oil (HCO); △, eicosapentaenoic acid/γ-linolenic acid (EPA/GLA); ■, EPA; □, GLA; ●, Chow. Diets fed continuously throughout the experiment are denoted by solid lines. Diets changed on day 7 are indicated by dotted lines. Each value represents the mean ± SD of at least three mice. Δ9 Desaturase activity at day zero: 180 ± 30 pmol/min per mg of microsomal protein. [a]Significantly different from 7-day values (panel A) or 0-day values (panel B) at $P < 0.01$.

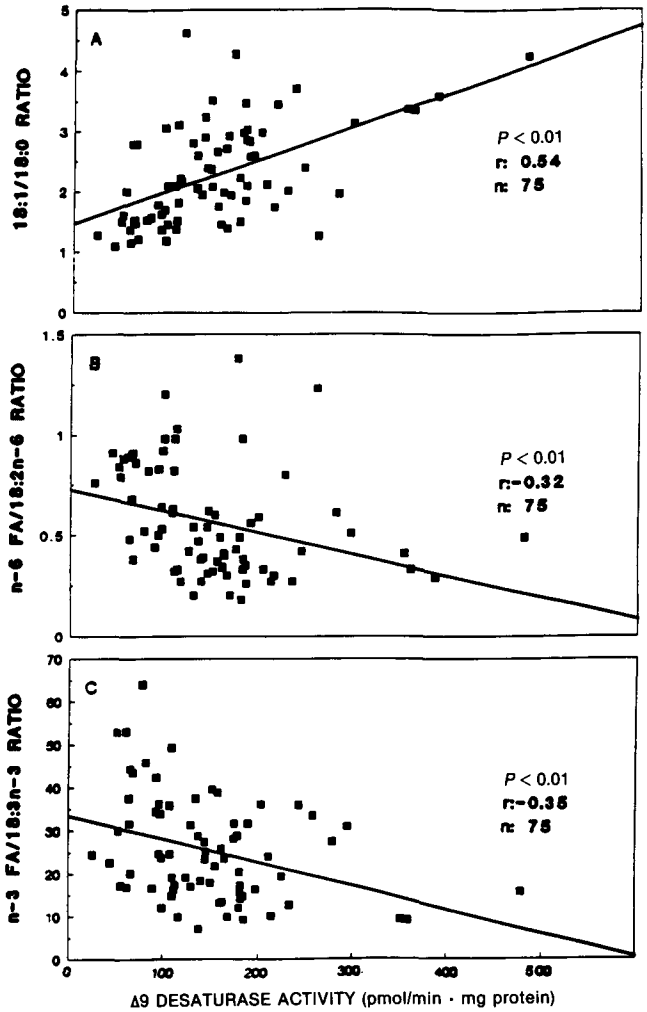

FIG. 4. Relationships between plasma fatty acids (FA) and hepatic Δ9 desaturase activity. Correlations were calculated by linear regression analysis as described in Figure 2. Δ9 Desaturase activity is expressed as pmol of substrate converted/min per mg of microsomal protein.

spective esters. Recently, Chautan *et al.* (24) have suggested that the plasma TG lowering effect of fish oil is partially due to incomplete hydrolysis of fish oil TGs by pancreatic lipase and reduced intestinal absorption of fatty acids. Therefore, we provided polyunsaturated n-3 and n-6 fatty acids in free fatty acid form in an attempt to maximize absorption. In the present study, the amounts of HCO or n-3/n-6 fatty acids added to the diets (2.5% by weight) were considerably lower than those used by other investigators (ranged from 8 to 20%) (9,25,26). Nevertheless, the dietary levels of n-3 and n-6 PUFAs were adequate to induce significant changes in the hepatic activity of Δ9 desaturase and several plasma lipid parameters (Figs. 1 and 3) without reducing the animals' body weight as reported elsewhere for higher fat intake (27).

The observed changes in plasma 18:1/18:0 ratio could not be attributed to differences in dietary 18:0 and 18:1 levels because the levels of 18:1 and 18:0 and the 18:1/18:0 ratios were similar in all diets tested (Table 1). Since the 18:1/18:0 ratio in plasma lipids was significantly and positively correlated with hepatic Δ9 desaturase activity (Fig. 4), it indicates that the plasma 18:1/18:0 ratio may reflect the liver Δ9 desaturase activity.

TABLE 3

Plasma TG Content and Liver Δ9 Desaturase Activity *vs.* Different Dietary PUFA Content[a]

	Dietary group			
	HCO	GLA	EPA	EPA/GLA
TG	2.0 ± 0.2	2.0 ± 0.5	1.5 ± 0.4[b]	0.9 ± 0.2[b,c]
Δ9	140.5 ± 27.5	139.0 ± 4.6	146.5 ± 10.6[b]	64.7 ± 4.1[b,c]
Dietary				
PUFA	29.2	57.8	54.9	61.2
P/S	0.6	2.8	2.9	3.4

[a]Each value represents the mean ± SD from three animals fed various supplemented diets for 21 d. TG, plasma triacylglycerol content, in mM. Δ9, Δ9 desaturase activity is expressed as pmol/min per mg microsomal protein. PUFA, percentage of total polyunsaturated fatty acids. P/S, polyunsaturated to saturated fatty acid ratio. For abbreviations, see Table 1.

[b,c]Significantly different ($P < 0.05$) from the HCO or EPA (or GLA) diet values, respectively.

It has been shown previously that dietary n-3 fatty acids can lower plasma TG levels and hepatic Δ9 desaturase activity (1,2,8). In the present study we observed that the ratios of post-Δ6 desaturation products to the parent acids in plasma lipids (n-3 fatty acids/18:3n-3 and n-6 fatty acids/18:2n-6) were negatively correlated with both plasma TG levels and Δ9 desaturase activity (Figs. 2 and 4). However, only the EPA/GLA diet was significantly effective in reducing both plasma TG levels and hepatic Δ9 desaturase activity when compared either to time zero values (Figs. 1 and 3) or to those from the HCO diet at the end of the study (Table 3). The EPA diet, when compared to the HCO diet at 21 d, caused a significant reduction in plasma TG levels but not in Δ9 desaturase activity (Table 3). These findings suggest that the TG and Δ9 desaturase activity-lowering effect of 20:5n-3, when given at low dose, requires the concomitant presence of n-6 PUFAs to be fully expressed.

These diet-induced differential effects may be related to the dietary PUFA levels and P/S ratio. Results shown in Table 3 indicated that increasing the PUFA content 2-fold and the P/S ratio 5-fold in the GLA diet, when compared to the HCO diet, did not affect plasma TG levels and Δ9 desaturase activities at the end of the study (21 d). These findings suggest that, under the experimental conditions used, the absolute PUFA levels or P/S ratios were not major factors in influencing plasma TG levels and Δ9 desaturase activity. On the other hand, the TG levels were reduced by 25% when animals were fed the EPA diet (Table 3) in spite of the fact that this diet had the same PUFA content and P/S ratio as the GLA diet. A further increase in PUFA content by only 11.5% and 17.2% in P/S ratio in the EPA/GLA diet significantly reduced both plasma TG and Δ9 desaturase activity by 55%. Again, this suggests that 20:5n-3 may lower TG levels and Δ9 desaturase activity only in combination with 18:3n-6. Whether these effects are confined to this strain of mouse or whether higher concentrations of 20:5n-3 in the diet could produce the changes documented here remains to be elucidated.

There are numerous mechanisms, such as modifications in peroxisomal and mitochondrial fatty acid oxidation (28,29) or in fatty acid and TG synthesis (30), that have been proposed to explain the effect of n-3 fatty acids on plasma TG levels. Insulin may also play a role in the TG-lowering effect of n-3 fatty acids. Otto *et al.* (25) have shown that n-3 fatty acid enriched diets decreased concomitantly both plasma insulin and TG levels. Insulin may also modify Δ9 desaturase activity and, consequently, the ratio of monoenoic to saturated fatty acids in the liver (31,32). In the present study, the levels of n-3 fatty acids were inversely related with the plasma TG levels as well as with the Δ9 desaturase activity (Figs. 2 and 4). These results are in agreement with the hypothesis that dietary n-3 fatty acids affect insulin levels or their receptors (25). However, it should be noted that the combined TG lowering and Δ9 desaturation suppressive effects of n-3 long chain fatty acids reported in this study were observed only in the presence of 18:3n-6. Since 18:3n-6 is an indirect precursor of eicosanoids (33), which are known to affect insulin secretion and binding (34–36), it is possible that this fatty acid may also contribute synergistically to the effects of n-3 fatty acids through insulin action. Additional investigations are needed to explore this possibility.

In summary, our data show that dietary supplementation with low doses of n-3 fatty acids (mainly 20:5n-3) and 18:3n-6 in combination, but not alone, caused a simultaneous reduction in plasma TG content and liver Δ9 desaturase activity. TG levels were positively correlated with the plasma 18:1/18:0 ratio but negatively correlated with the levels of n-6 and n-3 long chain PUFAs. It is suggested that these effects may be caused by a common factor (such as insulin) which is modulated by the presence of both 18:3n-6 and 20:5n-3 and/or their metabolites.

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Age-Related Changes in Δ6 and Δ5 Desaturase Activities in Rat Liver Microsomes

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Age-related changes in Δ6 desaturation of [1-^{14}C]α-linolenic acid and [1-^{14}C]linoleic acid and in Δ5 desaturation of [2-^{14}C]dihomo-γ-linolenic acid were studied in liver microsomes from Wistar male rats at various ages ranging from 1.5 to 24 mon. Desaturase activities were expressed both as specific activity of liver microsomes and as the capacity of whole liver to desaturate by taking into account the total amount of liver microsomal protein. Δ6 Desaturation of α-linolenic acid increased from 1.5 to 3 mon and then decreased linearly up to 24 mon to reach the same desaturation capacity of liver measured at 1.5 mon. The capacity of liver to desaturate linoleic acid increased up to 6 mon and then remained constant, whereas microsomal specific activity was equal at 1.5 and 24 mon of age. The capacity of liver to convert dihomo-γ-linolenic acid to arachidonic acid by Δ5 desaturation decreased markedly from 1.5 to 3 mon. It then increased to reach, at 24 mon, the same level as that observed at 1.5 mon. Age-related changes in the fatty acid composition of liver microsomal phospholipids at the seven time points studied and of erythrocyte lipids at 1.5 and 24 mon were consistent with the variations in desaturation capacity of liver. In particular, arachidonic acid content in old rats was slightly higher than in young rats whereas contents in linoleic and docosahexaenoic acids varied little throughout the life span. The results suggest that, in liver, the activity of desaturases may be regulated in the course of aging to maintain a constant level of polyunsaturated fatty acids in cellular membranes.
Lipids 28, 291-297 (1993).

Arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), which are the major polyunsaturated fatty acids of phospholipids in mammalian cells, are formed, principally in liver, from the precursors linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), respectively. The essentiality of these precursors depends on their capacity to be Δ6 desaturated and then transformed to 20:4n-6 and 22:6n-3 by chain elongation and Δ5 desaturation (20:4n-6), and by another elongation and Δ4 desaturation (22:6n-3). Desaturation and elongation are alternative reactions, but the desaturation processes are rate-limiting whereas the elongation steps are rapid (1-3). The hepatic Δ6 desaturase, which is the regulatory enzyme in the pathway involved in the biosynthesis of polyunsaturated fatty acids of the n-6 and n-3 series, converts 18:2n-6 to γ-linolenic acid (18:3n-6) and 18:3n-3 to stearidonic acid (18:4n-3). Another important enzyme, the Δ5 desaturase, is responsible for the biosynthesis of the major precursors of eicosanoids of series 2 and 3, *i.e.*, 20:4n-6 from dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-3) from eicosatetraenoic acid (20:4n-3). It also controls the balance between the precursors of eicosanoids of series 1 and 2 compounds (20:3n-6 and 20:4n-6, respectively), which have contrasting actions.

Several studies have shown that the *in vitro* fatty acid desaturase activities in liver microsomes are modified with aging. Peluffo and Brenner (4) showed that, in rat liver, Δ6 desaturation of 18:2n-6 and 18:3n-3 was decreased at 12 mon of age, when compared to desaturation at 3 mon. Bordoni *et al.* (5) demonstrated that the Δ6 desaturase activity toward 18:2n-6 decreased with the age of rats (between 1 and 22 mon). A linear correlation was proposed between activity and age. In another study, these authors showed that the decrease in the Δ6 desaturation of 18:3n-3 activity began later than did desaturation of 18:2n-6 (6). These results were obtained on Wistar rats fed a chow diet throughout their life. Choi *et al.* (7) found a significant age-dependent decrease in the Δ6 desaturation activity of 18:2n-6 between young (3-week-old) and adult (8-month-old) rats, but they did not confirm their results in a second study (8). Recently, Biagi *et al.* (9) showed that the Δ6 desaturation rate of 18:2n-6 in Wistar rats of 12 mon was only 44% of that found in young animals (3-month-old) after 1 mon of a semisynthetic diet containing soybean oil. The decrease with age in the rate of 18:3n-3 Δ6 desaturation was much less. Bourre *et al.* (10) found a 40% decrease in the Δ6 desaturation of 18:2n-6 in livers of mice between 4 and 17 mon. In studies using rat liver microsomes, the changes observed in the fatty acid compositions generally correlated with the impairment of Δ6 desaturation, suggesting a possible effect of age on the biosynthesis of prostaglandins and their metabolites.

These previous studies dealt only with Δ6 desaturation of 18:2n-6 or 18:3n-3 and often were limited to few time points. No study involved Δ5 desaturation. Moreover, results were expressed only as specific activity of Δ6 desaturation in liver microsomes. The Δ6 desaturation capacity of whole liver was not determined.

We therefore undertook a more complete survey of the evolution with age of Δ6 and Δ5 desaturation in rat liver. For this purpose, Wistar rats were fed the same balanced semisynthetic diet during their life span. The Δ6 desaturation of 18:2n-6 and of 18:3n-3 and the Δ5 desaturation of 20:3n-6 were determined in liver microsomes at seven time points ranging from 1.5 to 24 mon. Results were expressed both as specific activity and as total capacity of the liver to desaturate. The fatty acid composition of liver microsomal phospholipids was also determined to evaluate whether changes (if any) in the fatty acid profiles with age could be accounted for by modifications in desaturation specific activity or capacity. Data dealing with adult rats (3, 6, 9 mon of age) were reported in a preliminary study (11). The present paper describes the complete study carried out on young, adult and old rats.

MATERIALS AND METHODS

Animals and diet. Weanling male Wistar rats were randomized into groups of three rats and housed in stainless-steel cages in a well-ventilated room maintained at 22°C on a 12-h light/dark cycle. They were fed a balanced diet

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Abbreviation: Prot, protein.

containing (g/kg): casein, 220; DL methionine, 1.6; cellulose, 20; starch, 440; sucrose, 218; mineral mixture, 40; vitamin mixture, 10; oil mixture (50% palm oil plus 50% rapeseed oil), 50. In 100 g of diet, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) represented 945 and 188 mg, respectively (n-6/n-3 ratio, 5.0). The fatty acid composition of the diet lipids is shown in Table 1. The diet was stored at 4–6°C, and rats were fed *ad libitum*. Rats were maintained on this diet up to 1.5, 3, 6, 9, 12, 18 and 24 mon of age at the Laboratoire de Nutrition et Sécurité Alimentaire (INRA, Jouy en Josas, France). For the 1.5 mon study, only one group of six rats was used. For the other six ages (3 to 24 mon), two groups of three rats were used at two different periods (generally summer and winter) of the same year or of two different years in order to minimize the effect of season on the desaturation rate (4).

Chemicals. [1-^{14}C]Linoleic acid (58 mCi/mmol, 99% radiochemical purity) was purchased from CEA (Gif sur Yvette, France). [1-^{14}C]α-Linolenic acid and [2-^{14}C]dihomo-γ-linolenic acid (56 mCi/mmol, radiochemical purity, 96%) were purchased from the Amersham Radiochemical Centre (Amersham, United Kingdom). Each substrate was diluted in ethanol with unlabelled fatty acid to a specific activity of 10 mCi/mmol. Coenzymes, biochemicals and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of microsomal fractions. All experiments were performed starting at 7:30 a.m. to avoid any circadian variation in desaturase activity (12) and on night-fed animals in order to obtain the maximum desaturation activity (2).

The rats were anesthetized with sodium pentobarbital (6 mg per 100 g of body weight). They were exsanguinated by drawing blood from the abdominal aorta. The liver was excised, rinsed with ice-cold saline solution and weighed. About 3.5 g of liver were homogenized at 4°C in a Potter-Elvejhem homogenizer with 6 vol of 0.25 M sucrose in 0.05 M buffer phosphate (pH 7.4). The homogenate was centrifuged at 13000 × *g* (Model J-21B centrifuge, J-21 rotor, Beckman, Lyon, France) for 20 min to sediment cell fragments, mitochondria and nuclei. The supernatant was recentrifuged (L8-55 Ultracentrifuge, Ti 60 rotor, Beckman) at 105000 × *g* for 60 min, and the microsomal pellet was resuspended in 0.4 mL of supernatant and 0.8 mL of sucrose buffer. The amount of microsomal protein was determined by the method of Layne (13).

Desaturation assays. Incubations were performed in open flasks at 37°C for 15 min using a shaker water bath with a total volume of 2.1 mL incubation medium containing 72 mM phosphate buffer, pH 7.4, 4.8 mM $MgCl_2$, 0.5 mM coenzyme A, 3.6 mM adenosine 5′-triphosphate, 1.2 mM reduced nicotinamide adenine dinucleotide phosphate and 120 nmol, 120 nmol and 80 nmol of labelled 18:3n-3, 18:2n-6 and 20:3n-6 (30 μL of ethanolic solution), *i.e.*, about 57, 57 and 38 μM, respectively. At time 0, 5 mg of microsomal protein was added to the incubation medium. Incubations were stopped after 15 min by adding 15 mL of chloroform/methanol (1:1, vol/vol). Lipids were transmethylated in methanolic BF_3 according to Slover and Lanza (14).

The substrate and desaturation product were separated by high-performance liquid chromatography as described by Narce *et al.* (15), using a Waters Chromatograph Model 6000A and a Model 401 differential refractometer (Waters Associates, Milford, MA). The 250 × 4 mm i.d. column packed with LiChrospher 100 RP-18 (4 μm particles), protected by a LiChrosorb RP-18 precolumn, was purchased from Merck (Darmstadt, Germany). The fatty acid methyl esters were collected at the detector outlet, and their radioactivity was measured directly in the solvent by liquid scintillation counting with a Packard Model A 3000CD spectrometer (Packard Instruments, Rungis, France). The conditions permitted a good separation of the different polyunsaturated fatty acid methyl esters, and the desaturation product was eluted before its substrate, avoiding its contamination by the highly radioactive substrate.

Specific activity was expressed as pmol of radioactive fatty acid converted to desaturation product per min and per mg microsomal protein. For each animal, the desaturation capacity of whole liver was calculated by taking into account the total amount of microsomal protein in liver. It was expressed as nmol of substrate converted per min and per whole liver.

Preparation of erythrocytes. Erythrocytes were isolated from blood according to Rao *et al.* (16). Blood was collected from the abdominal aorta using heparin mixed with buffered saline solution (NaCl, 141 mM; KCl, 10 mM; $MgCl_2$, 1 mM; $CaCl_2$, 1.3 mM; NaH_2PO_4, 5 mM) in the proportion 1:5 (vol/vol). The suspension was gently shaken and centrifuged at 2500–3000 × *g* for 7 min. The pellet was resuspended in the saline solution and recentrifuged. The operation was repeated three times.

Fatty acid analysis. Lipids from an aliquot of liver microsomes and from the remaining pellet containing erythrocytes were extracted with dimethoxymethane/methanol (4:1, vol/vol) according to Delsal (17). Microsomal phospholipids were obtained by silicic acid column chromatography according to Hirsch and Ahrens (18). Fatty acids were transmethylated (14), and the fatty acid methyl esters were analyzed by gas-liquid chromatography using a Packard Model 417 chromatograph equipped with a laboratory made 30 m × 0.3 mm i.d. glass capillary column coated with Carbowax 20M. Analyses

TABLE 1

Fatty Acid Composition of Dietary Lipids[a]

Fatty acids	Mole %
14:0	0.2
16:0	7.7
16:1n-7	0.3
18:0	2.9
18:1n-9	57.3
18:1n-7	2.3
18:2n-6	20.1
18:3n-3	4.0
20:0	1.1
20:1n-9	1.5
22:0	1.8
24:0	0.8
Saturated	14.5
Monounsaturated	61.4
Polyunsaturated	24.1
n-6/n-3 ratio	5.0

[a]Mixture of rapeseed and peanut oils.

were carried out at 180°C at a nitrogen flow rate of 1 mL/min. The esters were detected with a flame-ionization detector. Peak areas were measured using a Delsi Model Enica 21 computing integrator (Delsi Instruments, Suresnes, France). Results are expressed as mole % of total fatty acids of the lipid fraction.

Statistical analysis. The results are expressed as means ±SD of six rats (1.5 mon) or of three rats in two different groups (3 to 24 mon). The graphs were traced using the means of the six rats for each age. After analysis of variance using the Fischer multiple range test, means were compared in the seven groups of age according to the least significant difference and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$). The fatty acid compositions of erythrocyte lipids and liver microsomal phospholipids were compared at 1.5 and 24 mon by means of Student's *t*-test.

RESULTS

Body weights, liver weights and liver microsomal protein concentrations at the seven time points studied are reported in Table 2. Body weight increased from 1.5 to 9 mon without any great difference between the two groups of three rats at 3, 6 and 9 mon of age. However, for the subsequent time points (12, 18 and 24 mon), body weight varied greatly between the two groups at each age. Liver weights also increased with age from 1.5 to 9 mon but more slowly than did body weights. As a consequence, the ratio of liver weight to body weight decreased from 1.5 to 6 mon. In the older rats, liver weight increased proportionally to body weight so that the liver weight/body weight ratio remained constant. The microsomal protein concentration was relatively constant, even in the two different groups (of the same age) of old rats so that the microsomal protein content of whole liver followed roughly the increase in liver weight.

The age-related specific activity of liver microsomes and capacity of whole liver to convert α-linolenic acid (18:3n-3) to stearidonic acid (18:4n-3) by Δ6 desaturation are reported in Figure 1. Both specific activity and liver capacity increased from 1.5 to 3 mon, but the latter increased more (2.8-fold) than did the former (1.3-fold). After 3 mon, the two parameters decreased with age but the specific activity decreased more rapidly than did liver capacity. At 24 mon, the specific activity of Δ6 desaturation of 18:3n-3 was 60% of that observed at 1.5 mon, while the liver capacity had returned to the initial level.

The parameters for the Δ6 desaturation of linoleic acid (18:2n-6) to γ-linolenic acid (18:3n-6) are reported in Figure 2. The specific activity of microsomes showed some variations with age, with relatively large differences occurring between groups at each age. However, the desaturation rate remained essentially constant between 1.5 and 24 mon. Paralleling the increase in total microsomal protein content, the liver capacity to desaturate linoleic acid increased from 1.5 to 6 mon, reaching a plateau between 6 and 24 mon. At 24 mon, the capacity was twice that measured at 1.5 mon.

The age-related specific activity of liver microsomes and the capacity of whole liver to convert dihomo-γ-linolenic acid (20:3n-6) to arachidonic acid (20:4n-6) by Δ5 desaturation are reported in Figure 3. The specific activity decreased markedly (6.5-fold) between 1.5 and 3 mon and then increased very slightly up to 24 mon to reach a value 2.5-fold lower than that at the earliest age studied. The capacity of the liver to desaturate also decreased between 1.5 and 3 mon but more slowly (2.4-fold) than did specific activity. From then on it increased, and, at 24 mon, the capacity of the liver to desaturate 20:3n-6 to 20:4n-6 was not significantly different from that found at 1.5 mon.

Figure 4 illustrates the age-related percentages of linoleic acid, of arachidonic acid, its product of Δ6 and Δ5 desaturations, and of docosahexaenoic acid in liver microsomal phospholipids. The percentage of 18:2n-6 did not change between 1.5 and 24 mon. The percentage of 20:4n-6 increased slightly, with some variations, reaching a higher value at 24 mon (32.2%) than at 1.5 mon (26.2%). Consequently, the 20:4/18:2 ratio increased from 3.4 (1.5

TABLE 2

Age-Related Changes in Body Weight, Liver Weight and Liver Microsomal Protein[a]

Age (mon)	n	Body weight (g)		Liver weight (g)		Microsomal protein (mg/g liver)	
1.5	6		150 ± 7[f]		6.8 ± 0.6[e]		16.1 ± 1.7[c]
3	3	340 ± 35		11.8 ± 0.8		18.6 ± 4.5	
	3	313 ± 9	327 ± 27[e]	11.2 ± 0.5	11.4 ± 0.7[d]	19.1 ± 5.4	19.1 ± 3.4[b]
6	3	393 ± 28		11.7 ± 0.7		19.3 ± 0.6	
	3	430 ± 5	411 ± 27[d]	13.7 ± 0.4	12.7 ± 1.2[c,d]	13.4 ± 0.1	15.4 ± 2.2[c]
9	3	448 ± 5		13.1 ± 0.4		15.1 ± 1.5	
	3	510 ± 36	479 ± 40[c,d]	15.3 ± 1.2	14.2 ± 1.5[b,c]	19.1 ± 2.3	17.1 ± 2.8[c]
12	3	600 ± 54		18.2 ± 1.3		17.5 ± 1.3	
	3	410 ± 23	505 ± 110[b,c]	12.4 ± 1.3	15.3 ± 3.6[b]	18.9 ± 2.7	18.2 ± 2.0[b,c]
18	3	563 ± 7		14.6 ± 1.0		17.4 ± 2.4	
	3	491 ± 37	527 ± 57[b,c]	13.2 ± 2.1	13.9 ± 1.7[b,c]	17.9 ± 1.9	17.9 ± 2.0[b,c]
24	3	608 ± 57		15.6 ± 1.3		17.3 ± 3.2	
	3	474 ± 21	540 ± 82[b]	12.6 ± 0.2	14.1 ± 1.9[b,c]	14.5 ± 0.9	15.8 ± 2.7[b,c]

[a]Results are means ± SD for n = three or six animals in each group. After analysis of variance (Fisher multiple range test), means of the six rats per age were compared in each column according to the least significant difference and classified in decreasing order. Means assigned different superscript letters (b–f) were significantly different ($P < 0.05$).

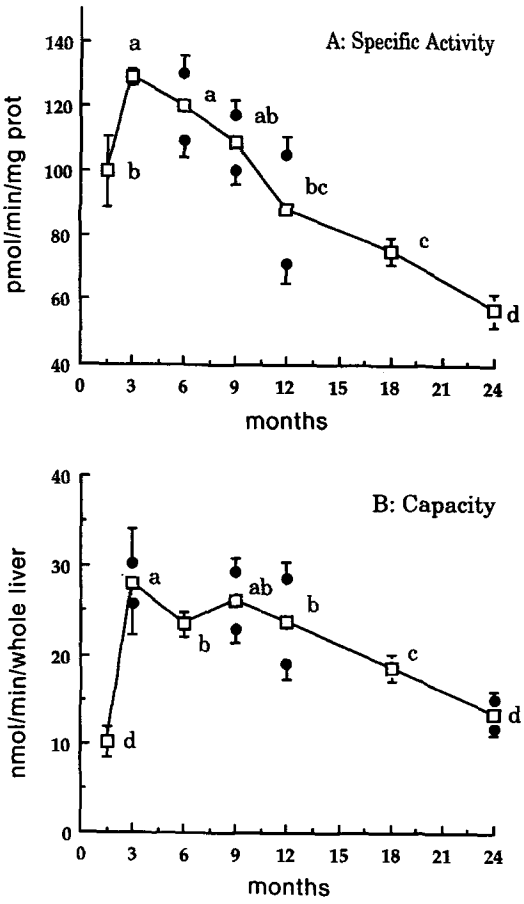

FIG. 1. Age-related changes in Δ6 desaturation of α-linolenic acid (18:3n-3) in liver microsomes expressed both as specific activity of liver microsomes (A) and activity of whole liver or capacity (B). Results are means ±SD obtained from one group of six rats (1.5 mon) or two groups of three rats at all other ages. After analysis of variance using the Fisher multiple range test, means of the six rats for each age were compared according to the least significant difference and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$). Prot, protein.

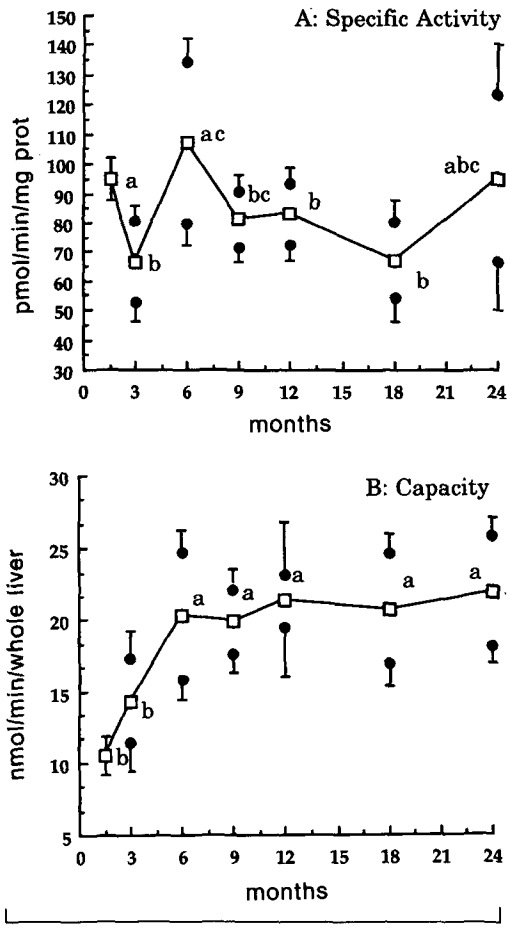

FIG. 2. Age-related changes in Δ6 desaturation of linoleic acid (18:2n-6) in liver microsomes expressed both as specific activity of liver microsomes (A) and activity of whole liver or capacity (B). Results are means ±SD obtained from one group of six rats (1.5 mon) or two groups of three rats at all other ages. After analysis of variance using the Fisher multiple range test, means of the six rats for each age were compared according to the least significant difference and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$). Prot, protein.

mon) to 3.8 (24 mon). The percentage of 18:3n-3 remained very low (*ca.* 0.3%) (unreported results) and constant, while that of 22:6n-3 decreased between 1.5 and 3 mon and then increased slightly to reach, at 24 mon, what had been the 1.5-mon value.

The fatty acid composition of erythrocyte lipids (mostly phospholipids) in 1.5- and 24-month-old rats is reported in Table 3, together with the composition of liver microsomal phospholipids at the same ages. The proportion of 18:2n-6 in erythrocytes was the same at the two ages studied, whereas the proportion of 20:4n-6 was higher in older rats. The percentage of 22:6n-3 in erythrocyte lipids was slightly lower in 24-month-old rats, but the level of total n-3 fatty acids was not significantly different at the two ages studied. The most striking difference in the fatty acid profiles of erythrocyte lipids and microsomal phospholipids is the higher percentage of 22:6n-3 and a lower percentage of palmitic acid (16:0) in the latter. Differences in the n-6 fatty acids were not pronounced.

DISCUSSION

The aims of this study were to investigate age-related changes in various desaturase activities of rat liver microsomes and to determine if these changes in turn induced alterations in the phospholipid fatty acid composition of erythrocytes and liver microsomes. The latter support the different desaturases and their associated proteins, and the former are rich in arachidonic acid.

Our data on the effects of age on Δ6 desaturation rates with 18:2n-6 and 18:3n-3 as substrates conflict with those reported by Hrelia *et al.* (6). These authors observed a decrease of 55% in the specific activity of Δ6 desaturase for 18:2n-6 between 1 mon and 22 or 25 mon. However, the Δ6 desaturation rate of 18:3n-3, much higher than that of 18:2n-6 at all ages, decreased from the end of 12 mon until 25 mon. We observed a pronounced decrease in the Δ6 desaturation of 18:3n-3 between 9 and 24 mon, whereas the Δ6 desaturation of 18:2n-6 was modified little through-

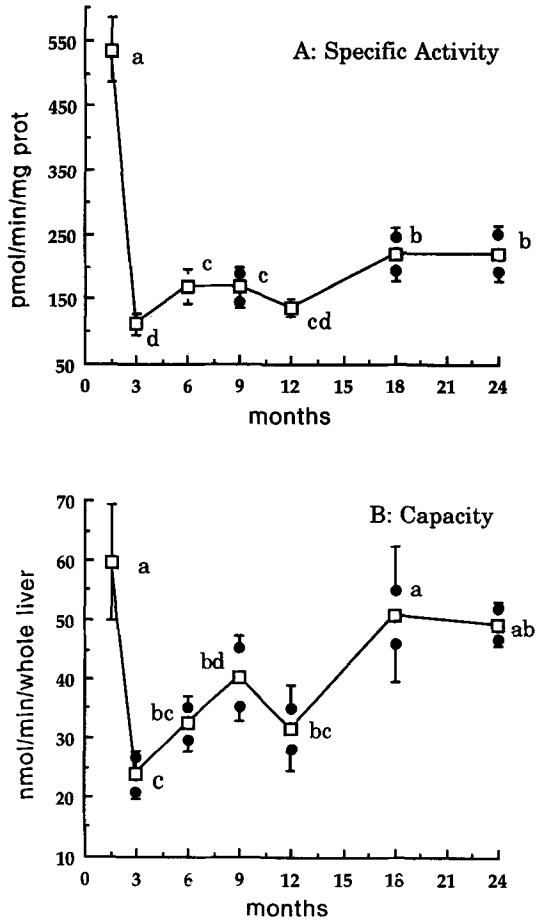

FIG. 3. Age-related changes in Δ5 desaturation of dihomo-γ-linolenic acid (20:3n-6) in liver microsomes expressed both as specific activity of liver microsomes (A) and activity of whole liver or capacity (B). Figures are means ±SD obtained from one group of six rats (1.5 mon) or two groups of three rats at all other ages. After analysis of variance using the Fisher multiple range test, means of the six rats for each age were compared to the least significant difference and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$). Prot, protein.

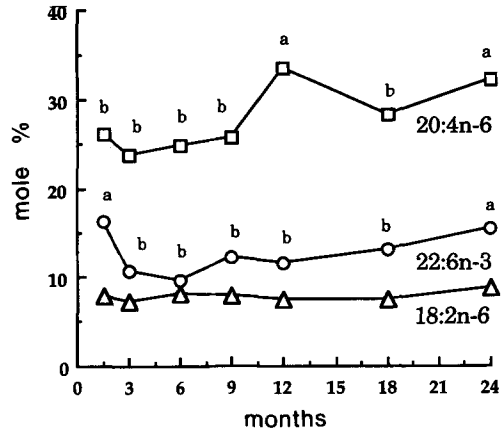

FIG. 4. Age-related changes in percentages of major polyunsaturated fatty acids in liver microsomal phospholipids. Results are means from six rats at each age. After analysis of variance using the Fisher multiple range test, the means were compared according to the least significant difference and classified in decreasing order. Means assigned different superscript letters were significantly different ($P < 0.05$).

out the life span. Moreover, the 18:2n-6 and 18:3n-3 Δ6 desaturation specific activities we observed at 1.5 mon (*ca.* 100 pmol/min/mg microsomal protein) were similar. Several differences between the experimental conditions used may in part explain these discrepancies. Hrelia *et al.* (6) used rats fed a chow diet throughout their life span and the liver microsomal preparations were frozen until use. Bourre *et al.* (10) also found a decrease with aging in Δ6 desaturation, but mice were used and desaturation measurements were done on liver homogenates. In our experiments, rats received a semisynthetic diet, and incubations were done immediately using fresh microsomes. In addition, we used a higher substrate concentration (57 μM instead of 40 μM used by Hrelia *et al.* (6)).

The differences in desaturation activities we observed between the two series of three animals, between 3 and 24 mon, were due in part to seasonal variations, as was previously shown for 6-month-old rats (11). To compensate for the seasonal variations of desaturation (4), we used two groups of animals at two different periods of the year. However, in the course of the experiment, another factor of variation appeared between the two groups: the liver weight in relation to the body weight. The desaturation rates varied in reverse order to the liver weight. So in 2-month-old rats, the amount of 18:2n-6 transformed by Δ6 desaturation was found equal to 71 ± 11 and 93 ± 4 pmol/min/mg microsomal protein in liver weighing 18.2 and 12.4 g, respectively. At certain ages, the effects of season and liver weight added and caused great variations between groups. At other ages, they compensated and the deviations were low. But within each group the variations between animals were not great.

No study has yet been reported on the age-related changes in Δ5 desaturase activity. Only data relative to the 20:3n-6/20:4n-6 ratio in liver lipids as a function of age have been reported, suggesting depressed Δ5 desaturase activity (19) or, on the contrary, a possible increase of this activity (20). Our results demonstrate that the specific Δ5 desaturase activity toward 20:3n-6 was 5.5 times higher than the Δ6 desaturase activity toward 18:2n-6 or 18:3n-3 at 1.5 mon.

The fatty acid composition of liver microsomal phospholipids and erythrocyte lipids reflected desaturation activities. The constant levels of 18:2n-6 and 20:4n-6 in liver microsomes after 3 mon are in agreement with the unchanged desaturation capacity of liver relative to 18:2n-6 and the increased capacity relative to 20:3n-6 in adult and aged animals. Despite some variations in desaturation activities, the 20:4n-6/18:2n-6 ratio varied little with age (3.4 to 3.8) except at 12 mon (4.5). These ratios were consistent with the observed changes in both Δ6 and Δ5 desaturation capacities. The changes in desaturation rates associated with the development of animals occur principally in the early stages of life.

The capacity of liver to convert 18:3n-3 to 18:4n-3 by Δ6 desaturation decreased with age, whereas the level of 22:6n-3 in microsomal phospholipids increased between 3 and 24 mon. An explanation for this may be that the decrease in Δ6 desaturase could be compensated for by

TABLE 3

Fatty Acid Composition (mole %) of Erythrocyte Lipids and Liver Microsomal Phospholipids at 1.5 and 24 Months of Age[a]

Fatty acids	Erythrocytes		Microsomes	
	1.5 mon	24 mon	1.5 mon	24 mon
14:0	1.0 ± 0.2	1.0 ± 0.2	0.6 ± 0.1	0.4 ± 0.1
16:0	27.2 ± 0.8	25.0 ± 0.2[b]	18.6 ± 1.0	16.2 ± 0.9[c]
16:1n-7	2.0 ± 0.9	2.1 ± 0.6	2.5 ± 0.6	2.0 ± 0.3
18:0	12.1 ± 0.5	15.6 ± 0.1[d]	13.6 ± 0.9	13.2 ± 2.5
18:1n-9	13.9 ± 0.7	12.1 ± 1.0[c]	9.7 ± 1.0	9.3 ± 1.2
18:2n-6	6.2 ± 0.1	5.9 ± 0.4	7.8 ± 0.9	8.6 ± 0.8
18:3n-6	0.6 ± 0.1	0.7 ± 0.3	0.3 ± 0.1	0.4 ± 0.1
18:3n-3	—	—	0.2 ± 0.1	0.2 ± 0.1
20:0	0.6 ± 0.1	0.5 ± 0.2	—	—
20:2n-6	0.6 ± 0.1	—	1.1 ± 0.3	0.3 ± 0.1[d]
20:3n-6	0.5 ± 0.1	0.8 ± 0.1[b]	1.3 ± 0.2	1.2 ± 0.2
20:4n-6	24.9 ± 1.2	26.9 ± 0.7[c]	26.2 ± 1.9	32.3 ± 1.3[d]
20:5n-3	0.4 ± 0.1	0.7 ± 0.4	1.2 ± 0.2	1.3 ± 0.4
22:0	0.7 ± 0.1	0.4 ± 0.2[c]	—	—
22:4n-6	1.8 ± 0.2	1.5 ± 0.1[b]	0.3 ± 0.1	0.2 ± 0.1
22:5n-6	—	—	1.1 ± 0.3	0.4 ± 0.2[d]
22:5n-3	1.7 ± 0.1	2.2 ± 0.2[c]	0.9 ± 0.2	0.4 ± 0.2[c]
22:6n-3	4.6 ± 0.4	3.3 ± 0.9[c]	12.9 ± 1.2	12.5 ± 1.1
24:0	0.5 ± 0.1	0.4 ± 0.2[c]	0.4 ± 0.4	—
total n-6	34.5 ± 1.1	35.8 ± 0.8[c]	38.0 ± 1.0	43.4 ± 2.0[d]
total n-3	6.7 ± 0.4	6.3 ± 1.5	15.2 ± 1.5	14.4 ± 1.5
n-6/n-3	5.2 ± 0.1	5.8 ± 1.0	2.5 ± 0.4	3.0 ± 0.4[c]

[a]In erythrocytes and microsomes means ± SD for n = six rats were compared between 1.5 and 24 mon using the Student's *t*-test.
[b]$P < 0.05$.
[c]$P < 0.01$.
[d]$P < 0.001$.

an increase in the Δ5 desaturation rate, as observed in the n-6 series, and/or by an increase in Δ4 desaturation and elongation rates, as reported recently by Mimouni *et al.* (21). On the other hand, an age-dependent decrease in the peroxisomal 22:6n-3 retroconversion, a decreased turnover rate and an increased incorporation into phospholipids may also explain that the 22:6n-3 levels in liver microsomes and in erythrocytes are maintained, despite a decrease in the Δ6 n-3 desaturation rate.

Although Δ6 and Δ5 desaturation activities are low in human liver (22–24), several studies indicated that few differences exist between elderly and young subjects with respect to the n-6 and n-3 fatty acid composition of serum (25,26), erythrocyte (27) and platelet (28) phospholipids. It can thus be assumed that in humans, as in rats, the desaturation activities are regulated to maintain constant the polyunsaturated fatty acid levels in membrane phospholipids. In particular, the desaturation rate could be increased to compensate for an increase, with aging, in peroxidation reactions in membranes. However, this compensation might disappear in different pathophysiological situations such as hypercholesterolemia or diabetes or modifications of nutritional conditions, leading to a decrease in polyunsaturated fatty acid content in tissue lipids and impairment of eicosanoid production.

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Effect of Δ^9-Tetrahydrocannabinol and Merthiolate on Acyltransferase Activities in Guinea Pig Liver Microsomes

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Δ^9-Tetrahydrocannabinol (THC) and merthiolate have been utilized as lysophospholipid acyltransferase inhibitors in metabolic studies. However, their effects on acyltransferases other than lysophosphatidylcholine:acyl-CoA acyltransferase (LPCAT) are not known. We have therefore investigated the effectiveness of THC and merthiolate in inhibiting the acylation of lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol (LPI) and lysophosphatidic acid (LPA) in guinea pig liver microsomes using oleoyl-CoA and arachidonoyl-CoA as acyl donors. THC inhibited LPCAT and lysophosphatidylethanolamine: acyl-CoA acyltransferase (LPEAT) by 40–50%, but had no effect or only slightly increased the activities of the other acyltransferases when assayed with oleoyl-CoA as the acyl donor. The results obtained with arachidonoyl-CoA were similar to those with oleoyl-CoA, with the exception of a 40% inhibition of lysophosphatidylserine:acyl-CoA acyltransferase (LPSAT) at concentrations of 50 μM or higher. At similar concentrations, merthiolate was more effective than THC in inhibiting the acyltransferases examined. Selective effects on the acyltransferases were observed at low concentrations of merthiolate (20 μM or less). Thus, LPCAT was most susceptible, followed by LPI acyltransferases, LPSAT, LPEAT and lysophosphatidic acid:acyl-CoA acyltransferases (LPAAT). The presence of LPA did not affect the inhibition of LPCAT by merthiolate. Thus the resilience of LPAAT to merthiolate inhibition was not due to chelation of the compound by the acidic lysolipid. Thiol reagents including *N*-ethylmaleiamide, 5,5′-dithio-*bis*-nitrobenzoic acid, iodoacetate, β-mercaptoethanol and dithiothreitol had little or no effect on the acyltransferases relative to equimolar concentrations of merthiolate. The above results indicate that merthiolate is a much more effective inhibitor of lysophospholipid:acyl-CoA acyltransferases than is THC, and that the selectivity exhibited by merthiolate may be due to direct and specific interaction with the acyltransferases. *Lipids 28,* 299–303 (1993).

A limiting step in the synthesis of eicosanoids and related compounds is the availability of unesterified fatty acids (1). Although these fatty acids are released from phospholipids by the action of phospholipases, increasing evidence suggests that acyltransferases play a key role in controlling the quantities of fatty acids available for eicosanoid synthesis (2–4). In order to investigate the effectiveness of agonists in stimulating the release of fatty acids or quantitation of fatty acids released, it is desirable to inhibit the enzymes that metabolize the released fatty acids, namely, the cyclooxygenases, lipoxygenases and acyltransferases. Inhibitors are available to block the cyclooxygenase and lipoxygenase pathways (5). A number of compounds, including some local anesthetics, have been shown to inhibit lysophosphatidylcholine:acyl-CoA acyltransferase (LPCAT) *in vitro* (6–8). Some of these also appear to inhibit LPCAT *in vivo* (6), but the effect of these inhibitors on the acylation of other lysophospholipids *in vitro* or *in vivo* is not known. A recent study reported the inhibition of LPCAT and lysophosphatidylethanolamine:acyl-CoA acyltransferase (LPEAT) by methyl lidocaine *in vitro* and in the isolated perfused hamster heart, but only moderate inhibition was obtained with a relatively high concentration (2 mM) of the local anaesthetic (8).

Agents generally regarded as acyltransferase inhibitors *in vivo* are Δ^9-tetrahydrocannabinol (THC) (9–12) and the thiol reagents merthiolate (thimerosal, 4) and *p*-hydroxymercuribenzoate (2,3). THC (10 μM) inhibited LPCAT activity by 80% in lymphocyte membranes (9). Merthiolate was equally effective in inhibiting LPCAT in macrophage membranes (4), but a higher concentration of *p*-mercuribenzoate (100 μM) was required to achieve a similar effect on the enzyme in macrophages, human lung fibroblasts or mouse liver membranes (2,3). Although these compounds may be effective inhibitors of LPCAT, this does not guarantee their effectiveness against other lysophospholipid:acyl-CoA acyltransferases. In the absence of such information, their utilization as general acyltransferase inhibitors may be inappropriate. Nevertheless, THC for example, is frequently cited as an acyltransferase inhibitor without mention of its possible limitations, despite conflicting reports on its effectiveness in inhibiting LPCAT *in vivo* (3,11,13). In view of this, the present study was undertaken to investigate the effect of THC and merthiolate on the acyltransferases involved in the acylation of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS) and lysophosphatidic acid (LPA) to form the parent phospholipids in guinea pig liver membranes. The specificity of merthiolate in inhibiting acylation processes was also investigated by comparing its effectiveness to that of other thiol reagents.

MATERIALS AND METHODS

Oleoyl-CoA, arachidonoyl-CoA, dimethylformamide (DMF), iodoacetate, *N*-ethylmaleiamide (NEM), 5,5′-dithio-*bis*-nitrobenzoic acid (DTNB), β-mercaptoethanol and merthiolate were obtained from Sigma Chemical Co. (St. Louis, MO). LPC (pig liver), LPE (pig liver), LPS (beef brain), LPI (pig liver) and LPA (egg lecithin) and

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Abbreviations: DMF, dimethylformamide; DTNB, 5,5′-dithio-*bis*-nitrobenzoic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid:acyl-CoA acyltransferase; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine:acyl-CoA acyltransferase; LPE, lysophosphatidylethanolamine; LPEAT, lysophosphatidylethanolamine:acyl-CoA acyltransferase; LPI, lysophosphatidylinositol; LPIAT, lysophosphatidylinositol:acyl-CoA acyltransferase; LPS, lysophosphatidylserine; LPSAT lysophosphatidylserine:acyl-CoA acyltransferase; NEM, *N*-ethylmaleiamide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; THC, Δ^9-tetrahydrocannabinol; TLC, thin-layer chromatography.

phospholipid standards were purchased from Serdary Research Laboratories (London, Ontario, Canada). [1-^{14}C]-Oleoyl-CoA and [1-^{14}C]arachidonoyl-CoA were the products of Du Pont (Mississauga, Ontario, Canada). Tetrahydrocannabinol was generously provided by Health and Welfare Canada (Ottawa, Ontario, Canada). Guinea pigs (250–300 g) were obtained from Charles River Canada Inc. (St. Constant, Quebec, Canada). Dithiothreitol (DTT) was purchased from BDH (Toronto, Ontario, Canada). Whatman K6 thin-layer chromatographic (TLC) plates and all other chemicals and solvents (reagent grade) were obtained from Baxter Canlab (Winnipeg, Manitoba, Canada).

Preparation of microsomes. Microsomes were prepared from guinea pig liver by differential centrifugation (14) and the protein content was measured by the method of Lowry *et al.* (15).

Acyltransferase assays. LPCAT was assayed in a reaction mixture containing 100 mM Tris-HCl (pH 8.5), 92 μM acyl-CoA (specific radioactivity of oleoyl-CoA, 0.6 μCi/μmol; arachidonoyl-CoA, 1.5 μCi/μmol), 142 μM LPC and 50 μg guinea pig liver microsomal protein in a total volume of 500 μL. Microsomes in buffer and water were preincubated at room temperature for 20 min with the drugs prior to the addition of the lysophospholipid and radiolabelled acyl-CoA to initiate the reaction. THC was dissolved in DMF, and the required concentrations were added in a volume of 5 μL. Control tubes had 5 μL DMF. Incubation was for 20 min at 25°C. Lysophosphatidylinositol:acyl-CoA acyltransferase (LPIAT), lysophosphatidylserine:acyl-CoA acyltransferase (LPSAT) and lysophosphatidic acid:acyl-CoA acyltransferase (LPAAT) were assayed with 142 μM of the appropriate lysophospholipid in place of LPC above. The concentration of acyl-CoA required for optimal activity of LPIAT, LPSAT and LPAAT was 46 μM. LPEAT was assayed with 46 μM acyl-CoA and 214 μM LPE. These conditions were established as optimal for measuring this activity. In experiments to investigate the effect of LPA on LPCAT activity, the required quantity of LPA was added to the assay after the 20 min preincubation with merthiolate prior to the addition of LPC and initiation of the reaction by the addition of radiolabelled acyl-CoA. All reactions were stopped by the addition of 3 mL chloroform/methanol (2:1, vol/vol) followed by 1 mL KCl (0.9%). After mixing, the two phases were clarified by centrifugation. Aliquots of the lower phase were removed for TLC analysis. All phospholipids were separated on activated Whatman K6 plates with the solvent system chloroform/methanol/water/acetic acid (50:37.5:2:3.5, by vol; Ref. 16). The radioactivity associated with the phospholipid bands was measured by scintillation counting using a Beckman LS 3801 Scintillation Counter (Palo Alto, CA).

Statistics. Statistical significance of differences was assessed by comparing the means by Student's *t*-test. Differences were considered to be significant if $P < 0.01$.

RESULTS

The specific activities of the enzymes in guinea pig liver microsomes that catalyze the acylation of LPC, LPE, LPS, LPI and LPA were measured with optimal concentrations of oleoyl- or arachidonoyl-CoA as acyl donors (Table 1). All these enzyme activities were present in the microsomal fraction. The activities of LPCAT, LPEAT, LPSAT and LPIAT were higher with arachidonyl-CoA as the acyl donor than with oleoyl-CoA. The only exception was LPAAT, which was 1.3-fold higher with oleoyl-CoA than with arachidonoyl-CoA. This observation may reflect a functional difference between the LPAAT, which is involved in *de novo* phospholipid biosynthesis *vs.* the other activities which are involved in the remodelling pathways responsible for the asymmetric distribution of polyunsaturated fatty acids at the glycerol *sn*-2 position of phospholipids (17).

The effects of THC on the acyltransferase activities were assessed with oleoyl-CoA and arachidonoyl-CoA as acyl donors (Table 2). The activity of LPCAT assayed with oleoyl-CoA was not affected by up to 20 μM THC. At 50 μM THC the activity was inhibited 50% with no further decrease at 100 μM. The effect of THC on the acylation of LPC with arachidonoyl-CoA was similar to those obtained with oleoyl-CoA. The lack of inhibition of LPCAT activity at low concentration of THC (10 μM) is at odds with previous reports of severe inhibition of LPCAT by 10 μM THC (9,18). Therefore, we investigated whether this might be due to the different pre-incubation conditions used in the current and in previous (9,18) studies. The effect of 10 μM THC on guinea pig heart microsomal LPCAT was investigated by pre-incubating the microsomes for 30 min at 37°C with THC dissolved in dimethylsulfoxide. Under these conditions the LPCAT activity was till unaffected.

LPEAT activity assayed with oleoyl-CoA or arachidonoyl-CoA as the acyl donor was unaffected by 10 μM

TABLE 1

Specific Activities of Acyltransferases in Guinea Pig Liver Microsomes with Oleoyl-CoA or Arachidonoyl-CoA as Acyl Donors[a]

Acyl donor	Acyl acceptor				
	LPC	LPE	LPS	LPI	LPA
18:1-CoA	152 ± 23	302 ± 32	199 ± 29	244 ± 30	694 ± 82
20:4-CoA	1337 ± 92	598 ± 61	625 ± 123	613 ± 66	528 ± 48

[a]Acyltransferase activities were measured using acyl acceptors and acyl donors as indicated. The concentrations of substrates for each acyltransferase are indicated in the Materials and Methods section. The specific activities are expressed in nmol phospholipid formed/h/mg protein. The values represent the means ± SD of three experiments each carried out in duplicate. LPC, lysophosphatidylcholine; LPC, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; LPI, lysophosphatidylinositol; LPA, lysophosphatidic acid.

TABLE 2

Effect of Δ^9-Tetrahydrocannabinol (THC) on Acyltransferase Activity with Oleoyl-CoA or Arachidonoyl-CoA as Acyl Donors

	Acyl-CoA acyltransferase activities (% control)[a]									
	LPC		LPE		LPS		LPI		LPA	
[THC]μM	18:1	20:4	18:1	20:4	18:1	20:4	18:1	20:4	18:1	20:4
0	100	100	100	100	100	100	100	100	100	100
10	103 ± 6	102 ± 2	96 ± 6	106 ± 4	132[c] ± 2	107 ± 3	98 ± 8	99 ± 5	106 ± 4	106 ± 7
20	93 ± 8	nd[b]	89[c] ± 2	nd	132[c] ± 5	nd	90 ± 6	nd	110[c] ± 3	nd
50	45[c] ± 5	42[c] ± 6	47[c] ± 7	63[c] ± 13	128[c] ± 2	58[c] ± 2	100 ± 4	95 ± 4	109[c] ± 4	112 ± 15
100	47[c] ± 7	31[b] ± 4	40[c] ± 2	28[c] ± 5	132[c] ± 5	55[c] ± 5	104 ± 6	82[c] ± 3	112[c] ± 7	154[c] ± 10

[a]Values represent the mean ± SD of three independent experiments carried out in triplicate.

[b]nd, Not determined. Abbreviations as in Table 1.

[c]Indicates values with significant differences from controls with a P value of at least $P < 0.01$.

THC, but increasing concentrations of the drug resulted in a progressive decrease in activity until, at a concentration of 100 μM, the activity was inhibited by 60% (Table 2). LPSAT activity was activated by approximately 30% at all concentrations of THC examined with oleoyl-CoA as the acyl donor. In contrast, LPSAT assayed with arachidonoyl-CoA was unaffected by 10 μM THC but was inhibited about 40% at concentrations of 50 μM or higher. Acylation of LPI was unaffected by the concentration range of THC used in our studies, irrespective of the acyl donor. LPAAT activity was either activated (10–50%) or not affected by THC with either oleoyl-CoA or arachidonoyl-CoA as acyl donor.

The effect of merthiolate on the acyltransferases was investigated in a fashion similar to that of THC using oleoyl-CoA or arachidonoyl-CoA as acyl donors (Table 3). The degree of inhibition of LPCAT by merthiolate was similar with either oleoyl- or arachidonoyl-CoA. At a concentration of 10 μM, about 90% of the activity was inhibited. LPIAT was also quite sensitive to the inhibitory effect of merthiolate irrespective of the acyl donor used in the assay. In contrast to LPCAT or LPIAT, acylation of LPS with both oleoyl- or arachidonoyl-CoA was relatively resilient to inhibition by merthiolate at low concentrations of the drug (<20 μM), but greater than 90% inhibition of LPSAT activity was observed in experiments with 100 μM merthiolate. LPEAT and LPAAT were also quite resistant to inhibition by merthiolate, especially the latter activity. A differential effect of merthiolate on LPEAT and LPAAT with different acyl donors was observed at the lower concentrations of the drug. LPEAT assayed with oleoyl-CoA was inhibited 30% by 10 μM merthiolate while acylation with arachidonoyl-CoA was inhibited 70% by the same concentration of merthiolate. The degree of inhibition obtained with both acyl donors was similar at higher concentrations of merthiolate. A modest activation of LPAAT (10–20%) was observed with merthiolate concentrations of 10 or 20 μM when arachidonoyl-CoA was used as the acyl donor. Acylation of LPA with oleoyl-CoA was inhibited about 15% at the same concentrations of merthiolate. LPAAT assayed with either acyl donor was inhibited between 60–70% by 100 μM merthiolate. One possible explanation for the inability of low concentrations of merthiolate to inhibit LPAAT could be the binding of the mercurial group of merthiolate to the negatively charged LPA. Such a reaction would decrease the effective concentration of merthiolate in the assay. To investigate this possibility, we examined the effect of varying concentrations of LPA on inhibition of LPCAT by merthiolate. The results which are displayed in Table 4 show no effect of LPA on the inhibition of LPCAT by merthiolate.

We also investigated the specificity of merthiolate in inhibition of the acyltransferases by comparing its effects with those of equimolar concentrations (20 μM) of the following thiol reagents: NEM, DTNB, iodoacetate, β-mercaptoethanol and DTT (Table 5). Merthiolate was clearly the most effective inhibitor of all the acyltransferase

TABLE 3

Effect of Merthiolate on Acyltransferase Activities with Oleoyl-CoA or Arachidonoyl-CoA as Acyl Donors

	Lysophospholipid:acyl-CoA acyltransferase activities (% control)[a]									
	LPC		LPE		LPS		LPI		LPA	
[Merthiolate]μM	18:1	20:4	18:1	20:4	18:1	20:4	18:1	20:4	18:1	20:4
0	100	100	100	100	100	100	100	100	100	100
10	11[b] ± 2	12[b] ± 4	67[b] ± 7	33[b] ± 4	40[b] ± 3	42[b] ± 6	16[b] ± 2	15[b] ± 4	83[b] ± 5	123[b] ± 7
20	4[b] ± 1	4[b] ± 2	36[b] ± 4	26[b] ± 2	34[b] ± 7	34[b] ± 5	7[b] ± 3	7[b] ± 1	87[b] ± 6	113[b] ± 5
50	3[b] ± 2	1[b] ± 1	32[b] ± 6	15[b] ± 6	23[b] ± 4	25[b] ± 6	6[b] ± 4	3[b] ± 2	39[b] ± 10	62[b] ± 9
100	2[b] ± 1	2[b] ± 1	21[b] ± 5	18[b] ± 4	8[b] ± 2	5[b] ± 1	4[b] ± 2	3[b] ± 1	37[b] ± 9	31[b] ± 6

[a]Values represent the means ± SD of three independent experiments carried out in triplicate. Abbreviations as in Table 1.

[b]Indicates values with significant differences from controls with a P value of at least $P < 0.01$.

TABLE 4

Effect of LPA on Inhibition of LPCAT Activity by Merthiolate

Additives	LPCAT activity (% control)[a]
None	100
20 μM Merthiolate	8 ± 4
20 μM Merthiolate + 35 μM LPA	5 ± 2
20 μM Merthiolate + 71 μM LPA	4 ± 2
20 μM Merthiolate + 142 μM LPA	3 ± 1

[a]Values represent the mean ± SD of two independent experiments carried out in triplicate. LPA, lysophosphatidic acid; LPCAT, lysophosphatidylcholine:acyl-CoA acyltransferase.

reactions. Little inhibition of any acyltransferase activity was observed with the five compounds examined with the exception of a 24 and 37% decrease in LPCAT and LPSAT, respectively, by NEM and a 12–16% decrease in LPCAT and LPSAT by DTNB.

DISCUSSION

In this study we have examined the effectiveness of THC and merthiolate on the acylation of LPC, LPE, LPS, LPI and LPA in guinea pig liver microsomes. The results clearly demonstrate that THC does not inhibit all microsomal acyltransferases and even slightly activates some of the enzymes. Of those activities inhibited (LPCAT and LPEAT), high concentrations of 50 μM or more were required to achieve significant inhibition. The lack of inhibition of LPCAT at low concentrations of THC was surprising in view of the effectiveness of the drug in inhibiting the lymphocyte membrane enzyme (9). Although we cannot offer an explanation to account for the differences in effectiveness of THC in the present study and those of previous studies with lymphocyte membranes (9), the possibility that the LPCAT in different tissues may not be identical and may respond differently to the drug cannot be ruled out. Thus in rat islets, LPCAT activity was inhibited by only 20% after 30 min incubation with 20 μM THC (11). These values are in the range of those obtained in the present study rather than those reported for lymphocyte membrane (9).

It is also not clear why THC has a differential effect on the LPSAT activity assayed with oleoyl-CoA or arachidonoyl-CoA as acyl donors. Nevertheless, these results may be an indication that the drug may interact directly with the enzyme. If the effect was mediated through perturbation of the environment, one would not expect the effect of the drug to be dependent on the acyl donor. Another possible explanation is the existence of different enzymes that catalyze the acylation of LPS with different acyl donors (19).

Merthiolate proved to be a more effective inhibitor of acyltransferase reactions in the guinea pig liver microsomes than THC, resulting in greater inhibition of the enzyme activities at comparable concentrations of THC. At low concentrations (20 μM), merthiolate not only exhibited some specificity toward the different acyltransferases (LPCAT = LPIAT > LPSAT = LPEAT > LPAAT), but was also able to discriminate between the activities with different acyl donors for LPA and LPE. These observations were surprising as merthiolate is a sulfhydryl reagent and would be expected to react nonspecifically with the enzymes. The ineffectiveness of other sulfhydryl reagents in inhibiting the various acyltransferase activities suggests that the effectiveness of merthiolate may be unrelated to its reducing properties. In macrophages, *N*-ethylmaleiamide was unable to inhibit LPCAT under conditions where merthiolate (10 μM) inhibited the activity by 70% (4). The differential sensitivity of various acyltransferases to merthiolate and the demonstration that this cannot be attributed to chelation of the drug by acidic lysolipids suggest that merthiolate may interact directly and specifically with the enzymes.

The inability of THC to inhibit some acyltransferases and the moderate inhibition it had on other activities would suggest that if employed solely for the purpose of inhibiting the acylation of fatty acids released by agonists, a substantial amount of the released fatty acid could be reacylated in the presence of the drug, thereby resulting in an underestimation of the quantities of fatty acids released. On the other hand, low concentrations of

TABLE 5

Effect of Thiol Reagents on Acyltransferase Activities with Oleoyl-CoA as the Acyl Donor

Additive (20 μM)	Lysophospholipid:acyl-CoA acyltransferase activity (% control)[a]				
	LPC	LPE	LPS	LPI	LPA
None	100	100	100	100	100
Merthiolate[b]	4[c] ± 1	36[c] ± 4	34[c] ± 7	7[c] ± 3	87[c] ± 6
DTT	101 ± 7	110 ± 8	93 ± 2	102 ± 9	101 ± 4
β-Mercaptoethanol	104 ± 11	110 ± 7	103 ± 5	102 ± 15	96 ± 8
DTNB	88[c] ± 7	102 ± 8	84[c] ± 4	99 ± 8	106 ± 3
Iodoacetate	99 ± 12	103 ± 5	107 ± 8	108 ± 9	104 ± 5
NEM	76[c] ± 11	90 ± 8	63[c] ± 10	92 ± 10	105 ± 8

[a]Values represent the mean ± SD of two independent experiments carried out in triplicate. Abbreviations as in Table 1. DTT, dithioreitol; DTNB, 5,5′-dithio-*bis*-nitrobenzoic acid; NEM, *N*-ethylmaleiamide.

[b]Values for merthiolate were obtained from Table 3.

[c]Indicates values with significant differences from controls with a P value of at least $P < 0.01$.

merthiolate were very effective inhibitors of the acylation of LPCAT and LPIAT, and higher concentrations of merthiolate inhibited the activity of the other lysophospholipid:acyl-CoA acyltransferases *in vitro*.

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Cholesterol Gallstone Induction in Hamsters Reflects Strain Differences in Plasma Lipoproteins and Bile Acid Profiles

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Because different strains of hamsters vary in their susceptibility to gallstones, the relationship between plasma lipoproteins, hepatic cholesterol, bile lipids and bile acid profile was examined during gallstone induction in strains of male Syrian hamsters from Charles River Lakeview (CHR), Biobreeder F_1B (BIO) and Harlan Sprague-Dawley (HAR). Gallstones were induced by feeding a purified diet containing 0.4 or 0.8% cholesterol for 5 wk. Basal plasma total cholesterol was similar, but the hypercholesterolemia induced by dietary challenge was significantly lower in CHR than in HAR and BIO hamsters. Cholesterol-fed CHR hamsters transported cholesterol mainly in HDL (47%), whereas VLDL-C + IDL-C predominated in BIO and HAR hamsters, and their HDL transported only 28 and 38%, respectively. HAR hamsters accumulated the most hepatic cholesterol, revealed the highest cholate/cheno ratio, the lowest glycine/taurine ratio and hydrophobicity index. HAR also developed the fewest cholesterol gallstones (23%), while 64% of CHR and 58% of BIO hamsters had cholesterol gallstones and 34% of BIO hamsters developed pigment stones. Doubling dietary cholesterol from 0.4 to 0.8% doubled the incidence of cholesterol gallstones but exerted minimal impact on other parameters compared to strain differences. Thus, different strains of hamsters vary considerably with respect to biliary cholesterol, bile acid profile and formation of cholesterol gallstones associated with differences in plasma lipoprotein profiles.
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Syrian hamsters are commonly used as a model for human lipid metabolism and cholelithiasis because hamster cholesterol and bile acid metabolism have certain similarities to humans (1,2). Furthermore, the hamster model is useful because cholesterol gallstones are readily induced by dietary manipulations (3-9). In the original studies by Dam (3), gallstones were induced most consistently by feeding hamsters a fat-free, glucose-rich diet. Other diet models have evolved that include a modest level of butterfat with or without dietary cholesterol (4-9). Whereas Dam's model is associated with essential fatty acid deficiency and results in abnormal physiology, other diet models are nutritionally more adequate. Our current purified diets are fomulated with a modest level of fat (5%) and a moderate amount of dietary cholesterol (0.4%) (8).

A complicating factor in hamster gallstone studies is the high incidence of pigment stones, which seem to be enhanced by feeding hamsters purified diets (3,8,10). Hamsters from different commercial sources also differ in their susceptibility to cholesterol gallstones (6), and differences in the metabolism of cholesterol, hepatic and biliary lipids as well as lipoprotein cholesterol distribution have been reported for different strains (11-15). For example, the hybrid F_1B hamster (Biobreeder) develops a unique cholesterolemic response to dietary cholesterol and saturated fat that renders it susceptible to fatty streak formation (16,17). In addition, a purified cholesterol-free diet containing 5% fat as a standard American fat blend (18) produced differences in plasma and biliary lipids, including the bile acid profile, in four strains of hamsters [Charles River Lakeview (CHR), Biobreeder F_1B (BIO), Harlan Sprague-Dawley (HAR) and Sasco] commonly used to study lipid metabolism.

In previous studies (7,8), hamsters fed a cholesterol-supplemented diet developed lithogenic bile and cholesterol gallstones in association with a lipoprotein profile that was characterized by an elevated very low density lipoprotein (VLDL)-cholesterol pool. Whether or how plasma lipoproteins modulate biliary cholesterol saturation and bile acid composition in the pathogenesis of cholesterol gallstones is poorly understood (19). Further, despite the importance of the hydrophobic/hydrophilic character of individual bile acids on cholesterol solubility (20), biliary cholesterol and phospholipid secretion (21) and bile acid synthesis (22), the impact of the physicochemical properties of dihydroxy *vs.* trihydroxy bile acids on bile lipid changes and cholesterol gallstone formation are not well defined *in vivo*.

To further examine this relationship and to identify the most susceptible model, we challenged three strains of hamsters (CHR, BIO and HAR) with a gallstone-inducing diet providing 0.4 or 0.8% dietary cholesterol while assessing plasma, hepatic and biliary lipids with particular emphasis on the individual bile acid profile. Our hypothesis was that the relationship between plasma lipoproteins, hepatic cholesterol and the bile acid profile would differ substantially during gallstone induction in hamsters reported to differ in their susceptibility to gallstones (6,7,13)

MATERIALS AND METHODS

Animals and diets. Thirty-six golden Syrian hamsters *(Mesocricetus auratus),* approximately 25-35 d old weighing 50-70 g were obtained from three different sources, namely Charles River Breeding Labs [Wilmington, MA (Lakeview strain)], Biobreeder Inc. [Watertown, MA (Hybrid F_1B hamster)] and Harlan Sprague-Dawley (Indianapolis, IN). Animals in each strain were randomly assigned to two groups (n = 5-7) and fed either of two purified diets containing 5% butter and either 0.4 or 0.8% cholesterol (Table 1) for 5 wk. The composition of the diet was in g/kg dry weight: casein 200, cornstarch 350, glucose 200, cellulose 100, wheat bran 50, fat 50, Ausman-Hayes mineral mix 6, Hayes-Cathcart vitamin mix 12, cholesterol 4 or 8, respectively, and choline chloride 3. The composition of the mineral and vitamin mix were detailed previously (10). Hamsters were housed in groups of 3-5

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Abbreviations: BIO, Biobreeder F_1B strain; CHR, Charles River Lakeview strain; HAR, Harlan Sprague-Dawley strain; HDL, high density lipoprotein; HI, hydrophobicity index; HPLC, high-performance liquid chromatography; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LI, lithogenic index; TC, plasma total cholesterol; VLDL, very low density lipoprotein.

TABLE 1

Body Weight Gain and Liver, Cecal and Perirenal Adipose Fat Weight in Three Strains of Hamsters Fed Gallstone-Inducing Diets Containing 0.4% or 0.8% Dietary Cholesterol[a] for Five Weeks[b]

	CHR	BIO	HAR
Food received, g/d	----------25----------		
Food consumed, g/d	20 ± 3	19 ± 3	20 ± 3
Initial body weight, g	69 ± 4[c]	66 ± 2[d]	52 ± 3[c,d]
Terminal body weight, g	117 ± 10	110 ± 11	122 ± 15
Weight gain/day, g	1.5 ± 0.3[c]	1.3 ± 0.2[d]	2.2 ± 0.4[c,d]
Liver weight, % body wt	5.1 ± 0.2	4.7 ± 0.5	4.9 ± 0.5
Cecum weight, % body wt	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.2
Perirenal fat weight, % body wt	0.7 ± 0.1[c]	0.7 ± 0.1[d]	0.5 ± 0.1[c,d]

[a]Statistical analysis [two-factorial analysis of variance (ANOVA)] revealed no effect of dietary cholesterol so data were combined.

[b]Values are means ± SD for 11-13 hamsters. CHR, Charles River Lakeview strain; BIO, Biobreeder F_1B strain; HAR, Harlan Sprague-Dawley strain.

[c,d]Means sharing a common superscript are significantly different ($P < 0.05$).

animals per cage with a bedding of wood chips kept in a temperature-controlled environment with a 12-h light/dark cycle (lights on 18:00 h). Hamsters were fed starch-gel diets *ad libitum* (25 g wet wt per hamster daily), and the food intake was monitored on a daily basis. Water was provided *ad libitum*. Body weights were monitored on a weekly basis. All protocols and procedures were approved by the Brandeis University Animal Care and Use Committee, Waltham, MA.

Plasma lipids and lipoprotein analysis. Blood was drawn upon arrival (baseline) and at the end of 5 wk. Prior to blood sampling, hamsters were fasted overnight (18 h) individually in wire-bottomed cages. Blood samples were collected under light anesthesia into an ethylenediaminetetraacetic acid wetted syringe by cardiac puncture, and plasma was separated immediately by centrifugation at 12,000 × *g* for 5 min. Total plasma cholesterol, high density lipoprotein (HDL)-cholesterol and triglycerides were determined by enzymatic assays (Sigma kit No. 352 for cholesterol and No. 336 for triglycerides, Sigma Chemical, St. Louis, MO). HDL-cholesterol was assayed following Mg^{2+}-phosphotungstate precipitation of lipoproteins containing apo B and apo E using HDL-cholesterol Reagent Set® (Boehringer Diagnostics, Indianapolis, IN) according to the procedure described by Weingard and Daggy (23). At 5 wk, plasma from 2-3 hamsters from each strain with similar cholesterol and triglyceride concentrations were pooled for plasma lipoprotein isolation. Lipoproteins were separated by density-gradient ultracentrifugation (24) in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a SW-41 rotor at 35,000 rpm and 15°C for 24 h with each tube stained with minimal Sudan Black to visualize lipoproteins (25). Three lipoprotein fractions were isolated, VLDL plus intermediate density lipoprotein (IDL) ($d < 1.019$ g/L), low density lipoprotein (LDL) ($1.019 < d < 1.055$ g/L) and HDL ($1.055 < d < 1.21$ g/L). Each lipoprotein fraction was dialyzed extensively against 0.15 M NaCl (pH 7.4) containing 100 mM butylated hydroxytoluene. Cholesterol, triglycerides and phospholipids were determined using enzymatic assays (Sigma kit No. 352 for cholesterol and No. 336 for triglycerides and Wako phospholipids B kit for phospholipids, Wako Chemicals, Richmond, VA). Protein concentration was determined according to the Lowry procedure (26).

Necropsy and gallstone evaluation. After 5 wk, hamsters were exsanguinated under anesthesia, and the liver, cecum and perirenal adipose fat excised, blotted and weighed. A portion of each liver was removed and frozen for hepatic cholesterol analysis. Gallbladder bile was aspirated for analysis of biliary lipids and bile acids prior to inspection for gallstones. The gallbladder was dissected from the liver, opened under a dissecting microscope and examined along with the remaining gallbladder bile for gallstones under regular and polarized light by light microscopy as previously described (10).

Bile analysis. Bile lipids were isolated using a modified Folch extraction (27). Ten μL of gallbladder bile was extracted with 3 mL chloroform/methanol (2:1, vol/vol) and 0.75 mL of 1.12% KCl solution. Biliary cholesterol was measured using an enzymatic assay (Wako free cholesterol C kit, Wako Chemicals) in an aliquot of the evaporated chloroform layer redissolved in 2-propanol. Biliary phospholipid concentration was determined in diluted gallbladder bile (1:10) by an enzymatic/colorimetric procedure using the Wako phospholipids B kit. Biliary bile acid concentration was determined as conjugated bile acids using an isocratic high-performance liquid chromatography (HPLC) procedure adapted from Rossi *et al.* (28). The mobile phase consisted of methanol/0.01 M KH_2PO_4 (75:25, vol/vol) to which 4.2 mL/L of 5 N NaOH was added before the pH was adjusted to pH 5.35 by adding about 1 mL of 85% H_3PO_4. The individual bile acid profile was analyzed using an aliquot of the methanol/KCl layer from the Folch extract, evaporated under a stream of nitrogen and redissolved in the mobile phase. A standard containing 60 mmol/L of taurine and glycine conjugates of cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid (Sigma Chemical Co.) was used to calculate the individual bile acid concentrations. Total bile acid concentration was calculated as the sum of individual bile acids (taurine and glycine conjugates of cholate,

chenodeoxycholate and lithocholate) as determined by HPLC. Taurine and glycine conjugates of ursodeoxycholic acid were present only in trace amounts and therefore not included in the calculation.

The lithogenic index (LI) was calculated according to published procedures (29) based on the relative molar ratios of lipid components and total lipid concentration using a computerized version of cholesterol solubility (30,31). The bile acid hydrophobicity index (HI) for each bile sample was determined by multiplying hydrophobicity indices of individual bile acids (glycine and taurine conjugates) by the mol fraction of each bile acid and summing the products (32).

Hepatic cholesterol analysis. Hepatic cholesterol was extracted by grinding a 100-mg portion of liver with anhydrous sodium sulfate and extracting three times with chloroform/methanol (2:1, vol/vol). The liquid phase was evaporated to dryness and redissolved in chloroform. An aliquot was evaporated and redissolved in 2-propanol alcohol for analysis of free cholesterol by HPLC (33). Total cholesterol was determined enzymatically after hydrolysis with alcoholic KOH for 1 h as adapted from Tercyak (34), using the Wako free cholesterol C kit for cholesterol analysis.

Statistical analysis. Statistical differences between the three strains and the two diets were calculated using a two-factorial analysis of variance (ANOVA) followed by Scheffe's F-test utilizing a SuperANOVA statistical software package (Abacus Concepts, Inc., Berkeley, CA). When no significant diet effect was found, data from both diets were combined to evaluate strain differences.

RESULTS

Growth. All hamsters were healthy and showed normal daily weight gain. *Ad libitum* food consumption per hamster averaged 19.5 g per day (10.8 g dry weight) with no apparent difference between strains or the two diets. HAR hamsters began with a significantly lower body weight (Table 2), but final body weights were not different between the three strains and were not affected by the level of dietary cholesterol. Accordingly, HAR hamsters were considerably more feed-efficient and gained significantly more daily weight than CHR and BIO hamsters, but their perirenal fat weighed significantly less than that of the two other strains (Table 1).

Plasma lipids and lipoprotein profile. No apparent difference in plasma total cholesterol (TC) was found initially between the three hamster strains which had an average plasma cholesterol concentration of 149 ±32 mg/dL. However, BIO hamsters showed the lowest initial high density lipoprotein-cholesterol (HDL-C) (Table 2). Not surprisingly TC progressively increased in all hamsters fed the cholesterol-supplemented diets. After 5 wks, the TC differences between 0.4% *vs.* 0.8% dietary cholesterol was +18, +24, and −8%, respectively, for CHR, BIO and HAR. Since the dietary cholesterol effect was not significant, the cholesterol groups were combined to evaluate the strain effect. On this basis CHR hamsters revealed significantly lower TC and HDL-C concentrations. However, HDL-C expressed as percent of TC did not differ between strains.

Initial plasma triglyceride (TG) concentrations differed significantly between strains with the highest TG found in BIO hamsters, while HAR hamsters were intermediate and CHR hamsters were lowest (Table 2). After 5 wks, TG did not differ as a function of strain or level of dietary cholesterol, although CHR hamsters demonstrated somewhat lower values.

The distribution of cholesterol and TG in VLDL + IDL, LDL and HDL (pooled dietary data) revealed striking differences between strains (Table 3). Cholesterol-fed CHR hamsters transported most of their cholesterol in the HDL fraction (47 ± 3%), whereas BIO and HAR hamsters carried cholesterol mainly in the VLDL + IDL fraction (51 ± 3% and 46 ± 8%, respectively). The percent distribution of low density lipoprotein-cholesterol (LDL-C) did not differ significantly between the three strains (CHR, 18%; BIO, 21%; and HAR, 15%). The differences in the cholesterol distribution were further apparent in the LDL/HDL and the VLDL + IDL/HDL ratios (Table 3). BIO hamsters had a significantly higher LDL/HDL cholesterol ratio (1.9 times higher) than HAR and CHR hamsters and also had a significantly higher VLDL + IDL/HDL cholesterol ratio

TABLE 2

Plasma Lipid Concentration in Three Strains of Hamsters Fed Gallstone-Inducing Diets Containing 0.4% or 0.8% Dietary Cholesterol[a] for Five Weeks[b]

	CHR	BIO	HAR
Plasma cholesterol, mg/dL			
Initial	147 ± 16	146 ± 15	153 ± 50
5 wks	387 ± 78[c,d]	610 ± 198[c]	548 ± 120[d]
HDL-cholesterol, mg/dL			
Initial	93 ± 17[c]	61 ± 8[c,d]	87 ± 15[d]
5 wks	158 ± 29[a,b]	188 ± 36[a]	198 ± 40[b]
Triglycerides, mg/dL			
Initial	161 ± 33[c,d]	350 ± 67[c,e]	239 ± 55[d,e]
5 wks	470 ± 241	740 ± 540	696 ± 396

[a]Statistical analysis (two-factorial ANOVA) revealed no dietary cholesterol effect so data were combined. HDL, high density lipoprotein. See Table 1, footnote b for other abbreviations.

[b]Values are mean ± SD for 11–13 hamsters.

[c,d,e]Means sharing a common superscript are significantly different ($P < 0.05$).

TABLE 3

Lipoprotein Cholesterol and Triglyceride Concentrations Following Gradient Ultracentrifuge Separation of Plasma from Three Strains of Hamsters Fed Gallstone-Inducing Diets Containing 0.4 or 0.8% Cholesterol[a] for Five Weeks[b]

	CHR	BIO	HAR
		mg/dL plasma	
Cholesterol			
VLDL + IDL	141 ± 47[d,e] (35)[c]	368 ± 23[d] (51)	271 ± 77[e] (46)
LDL	70 ± 2[d] (18)	147 ± 11[d,e] (21)	88 ± 31[e] (15)
HDL	183 ± 28 (47)	200 ± 30 (28)	223 ± 20 (39)
LDL/HDL ratio	0.39 ± 0.06[d]	0.75 ± 0.17[d,e]	0.39 ± 0.11[e]
(VLDL + IDL)/HDL ratio	0.76 ± 0.19[d]	1.88 ± 0.40[d,e]	0.39 ± 0.11[e]
Triglycerides			
VLDL + IDL	429 ± 137 (92)	697 ± 6 (85)	698 ± 243 (91)
LDL	17 ± 3 (4)	93 ± 36 (12)	42 ± 40 (5)

[a]Statistical analysis (two-factorial ANOVA) revealed no effect of dietary cholesterol so data were combined. Abbreviations: See Table 1. VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

[b]Values are mean ± SD. Plasma from 2-3 hamsters was pooled for each lipoprotein isolation (n = 2-4).

[c]Values in parentheses represent percent distribution.

[d,e]Means sharing a common superscript are significantly different ($P < 0.05$).

which was 2.5 times higher than in CHR and 1.6 times higher than in HAR hamsters (Table 3).

Hepatic and biliary lipids. The level of added dietary cholesterol did not influence hepatic cholesterol accumulation, but total and esterified cholesterol concentrations in liver were significantly higher in HAR hamsters compared to CHR or BIO hamsters. Free cholesterol concentrations did not differ between the three strains of hamsters and accounted for 10-15% of the TC (Table 4).

The biliary lipid composition expressed as mol% of bile acids, phospholipids and cholesterol, was significantly affected by strain and level of dietary cholesterol (Table 5). Specifically, BIO hamsters had the highest mol% biliary cholesterol, and all hamsters fed 0.8% dietary cholesterol had approximately 30% more biliary cholesterol than hamsters fed 0.4% cholesterol. In like manner, HAR hamsters had the highest mol% bile acids when fed 0.4% dietary cholesterol, but this difference between strains disappeared at 0.8% dietary cholesterol. By contrast, mol% biliary phospholipids was only higher in CHR hamsters at 0.4% dietary cholesterol (Table 5). The LI was not affected by the level of dietary cholesterol, but BIO hamsters revealed the highest LI for both diets.

Bile acid profile. Total bile acid concentration (mM) was not affected by the level of dietary cholesterol, but at 0.4% cholesterol intake HAR hamsters had a significantly higher total bile acid concentration than either CHR or BIO hamsters (Table 5). The composition of individual bile acids expressed as percentage of the total bile acid concentration revealed considerable differences between the three hamster strains (Table 6) with HAR hamsters demonstrating the highest percentage of cholate (49 ± 8%) followed by BIO hamsters (40 ± 6%) and CHR hamsters (31 ± 7%). In contrast, total cheno was highest in CHR hamsters (61 ± 7%) but was similar (45 ± 7 and 42 ± 7%) in BIO and HAR hamsters. As a result, the cholate/cheno ratio was lowest in CHR hamsters and highest in the HAR hamsters. A significant strain effect existed also for taurocholate with the highest percentage in HAR hamsters, and lowest in CHR hamsters, whereas

TABLE 4

Hepatic Cholesterol Concentration in Three Strains of Hamsters Fed Gallstone-Inducing Diets Containig 0.4 or 0.8% Dietary Cholesterol[a] for Five Weeks

	CHR	BIO	HAR
Liver weight (g)	5.9 ± 0.5	5.2 ± 0.8	5.9 ± 0.7
		mg/g liver	
Total cholesterol	34 ± 9[c]	31 ± 8[d]	45 ± 7[c,d]
Free cholesterol	3.9 ± 0.4	4.5 ± 0.9	4.3 ± 0.4
Esterified cholesterol[b]	30 ± 9[c]	26 ± 7[d]	41 ± 7[c,d]

[a]Statistical analysis (two-factorial ANOVA) revealed no effect of dietary cholesterol so data were combined. Abbreviations: See Table 1.

[b]Calculated as the difference between total and free cholesterol. Values are mean ± SD for 11-13 hamsters.

[c,d]Means sharing a common superscript are significantly different ($P < 0.05$).

TABLE 5

Biliary Lipid Concentration of Gallbladder Bile from Three Hamster Strains Fed Gallstone-Inducing Diets Containing 0.4 or 0.8% Dietary Cholesterol for Five Weeks[a]

	Diet					
	0.4% cholesterol			0.8% cholesterol		
	CHR	BIO	HAR	CHR	BIO	HAR
			mM			
Bile acids	82 ± 12[e]	87 ± 17[f]	147 ± 51[e,f]	121 ± 37	111 ± 18	103 ± 35
Phospholipids	13 ± 3	12 ± 3	17 ± 5	19 ± 3	15 ± 2	16 ± 5
Cholesterol[b]	8 ± 2	9 ± 2	8 ± 2	12 ± 6	16 ± 4	10 ± 2
			mol%			
Bile acids[b]	79 ± 2[e]	80 ± 2[f]	85 ± 2[e,f]	79 ± 3	78 ± 3	79 ± 3
Phospholipids	13 ± 2[e]	11 ± 1[e]	10 ± 2	13 ± 2	11 ± 2	12 ± 1
Cholesterol[b]	8 ± 1[e]	9 ± 1[f]	5 ± 1[e,f]	7 ± 2[g]	12 ± 3[g]	9 ± 2
Total lipids, g/dL	5.3 ± 0.8[e]	5.6 ± 1.3[f]	8.8 ± 3[e,f]	7.9 ± 2.3	7.3 ± 1.0	6.7 ± 2.2
Lithogenic index	1.9 ± 0.4[e]	2.2 ± 0.3[f]	1.3 ± 0[e,f]	1.6 ± 0.4[g]	2.7 ± 0.7[g,h]	1.9 ± 0.6[h]
			Gallstone incidence			
Chol. stones/crystals	3/5 (60%)	2/6 (33%)	0/7	4/6 (67%)	4/6 (67%)	3/6 (50%)
Mixed stones	0/5	2/6[c] (33%)	0/7	0/6	1/6[d] (17%)	0/6
Pigment stones	0/5	1/6 (17%)	2/7 (29%)	0/6	1/6 (17%)	0/6
No stones	2/5 (40%)	1/6 (17%)	5/7 (71%)	2/6 (33%)	0/6	3/6 (50%)

[a]Values are mean ± SD for 5-7 hamsters. See Table 1 for abbreviations.

[b]Significant diet effect was observed.

[c]Mix of 8 pigment stones (0.2-0.8 mm) and cholesterol crystals.

[d]Mainly cholesterol (Chol.) stones and crystals with 3 pigment stones (0.2 mm).

[e,f,g,h]Means within a diet group sharing a common superscript are significantly different ($P < 0.05$).

TABLE 6

Bile Acid Profile in Gallbladder Bile from Three Hamster Strains Fed Gallstone-Inducing Diets Containing 0.4 or 0.8% Dietary Cholesterol[a] for Five Weeks[b]

	CHR	BIO	HAR
		% distribution	
Taurocholate	15 ± 4[c,d]	20 ± 4[c,e]	33 ± 6[d,e]
Glycocholate	16 ± 5	19 ± 5	16 ± 10
Taurocheno	40 ± 8	33 ± 7	38 ± 8
Glycocheno	21 ± 4[c,d]	12 ± 5[c,e]	4 ± 2[d,e]
Taurodeoxycholate	3 ± 2[c,d]	8 ± 2[c]	7 ± 3[d]
Glycodeoxycholate	1 ± 2	2 ± 1	1 ± 1
Taurolithocholate	0.5 ± 0.8[c]	1.4 ± 1.0[c,d]	0.5 ± 1.0[d]
Glycolithocholate	3 ± 2	4 ± 5	1 ± 2
		ratios	
Cholate/cheno	0.5 ± 0.2[c,d]	0.9 ± 0.3[c,e]	1.2 ± 0.4[d,e]
Glyine/taurine	0.7 ± 0.2[c]	0.6 ± 0.2[d]	0.3 ± 0.3[c,d]
Primary/secondary	12.4 ± 3.6[c]	6.1 ± 2.6[c,d]	11.5 ± 4.8[d]
Hydrophobicity index	0.37 ± 0.03[c]	0.34 ± 0.05[d]	0.27 ± 0.04[c,d]

[a]Statistical analysis (two-factorial ANOVA) revealed no effect of dietary cholesterol so data were combined. See Table 1 for abbreviations.

[b]Values are mean ± SD for 11-13 hamsters.

[c,d,e]Means sharing a common superscript are significantly different ($P < 0.05$).

glycocholate was not affected by strain. Taurocheno was the predominant bile acid and was unaffected between strains, whereas glycocheno was 5.2 times higher in CHR than in HAR hamsters and 1.7 times higher than in BIO hamsters. A higher percentage of taurodeoxycholate was observed in BIO and HAR hamsters compared to CHR hamsters. As a result, the ratio of primary to secondary bile acids was lower in BIO hamsters than in CHR or HAR hamsters.

A significant strain effect and a strain-diet interaction

were noted in the glycine/taurine conjugation pattern (Table 6). HAR hamsters had a significantly higher percentage of taurine-conjugated bile acids (78 ± 12%) than CHR and BIO hamsters (59 ± 6% and 63 ± 9%, respectively). Correspondingly, glycine conjugation was significantly lower in HAR hamsters (22 ± 12%) while CHR and BIO hamsters conjugated 41 ± 6% and 37 ± 9% of their bile acids with glycine. As a result, the glycine/taurine ratio was lowest in HAR hamsters, highest in CHR hamsters with BIO hamsters being intermediate.

The HI, a measure of the hydrophobic/hydrophilic balance of biliary bile acids, was lowest in HAR hamsters and significantly increased in both CHR and HAR hamsters.

Gallstone incidence. Examination of gallbladders after 5 wk revealed a striking difference in gallstone incidence attributed to both the strain of hamsters and the level of dietary cholesterol (Table 5). Overall, HAR hamsters proved least susceptible to cholesterol gallstones with only 3 of 13 hamsters (both diets combined) developing cholesterol stones and only when fed 0.8% dietary cholesterol. Two of 7 HAR hamsters fed 0.4% dietary cholesterol formed pigment gallstones. Cholesterol stones or crystals were present in 3 of 5 CHR hamsters fed 0.4% and in 4 of 6 CHR hamsters fed 0.8% dietary cholesterol. No pigment stones were found among CHR hamsters (Table 5). In BIO hamsters fed 0.4% cholesterol 2 of 6 hamsters developed pure cholesterol stones, whereas 3 of 6 hamsters formed pigment stones, two of which were mixed with cholesterol crystals. Feeding 0.8% dietary cholesterol increased the incidence of cholesterol stones among BIO hamsters with 5 of 6 hamsters developing cholesterol stones (in one case 3 small pigment stones were mixed with cholesterol stones and crystals) (Table 5).

DISCUSSION

Recently we described significant differences in plasma and biliary lipids and the bile acid profile in four strains of Syrian hamsters maintained on a similar, but cholesterol-free purified diet (18). For the current study three of these strains (CHR, BIO and HAR) were selected based on distinct differences in lipoprotein and biliary lipid composition detected during the previous study (18). Sasco hamsters, although susceptible to cholesterol gallstones (6,7), were not included because previous comparisons have found them to be slightly less susceptible than CHR hamsters under our conditions (7; unpublished observations). Challenges with a cholesterol-enriched diet have revealed differences in hepatic cholesterol accumulation and lipoprotein and bile acid profiles that appear to affect the incidence of cholesterol gallstones, with CHR hamsters being the most susceptible strain.

Liver cholesterol and gallstone incidence. Hepatic cholesterol accumulation was affected by strain. Surprisingly no substantial effect of dietary cholesterol (0.4 *vs.* 0.8%) was evident, suggesting that a plateau was reached with 0.4% dietary cholesterol or that the 5-wk period was too brief to allow a difference to develop. Although HAR hamsters accumulated significantly more hepatic cholesterol than BIO and CHR hamsters, the LI and incidence of cholesterol gallstones tended to be lowest in this strain. In contrast, CHR hamsters, with the lowest hepatic cholesterol concentration, developed the most cholesterol gallstones. Overall, no correlation existed between the hepatic cholesterol concentration (both free and total) and either the lithogenic index or the absolute (mM) or relative (mol%) biliary cholesterol concentrations. This corroborates previous findings (35–37) emphasizing the lack of relationship between hepatic cholesterol concentration and biliary cholesterol saturation. Thus, increased liver cholesterol accumulation can lead to supersaturated bile with minimal or no cholesterol gallstone formation, as demonstrated by HAR hamsters. On the other hand, the lack of hepatic cholesterol accumulation is typically found in the EFAD model where bile supersaturation and gallstone formation are prevalent (8).

Biliary lipids in relation to gallstones. Although all hamsters developed supersaturated bile and an LI exceeding 1.0, HAR hamsters maintained higher mol% bile acids and lower mol% biliary cholesterol resulting in the lowest LI. The LI of BIO hamsters was 1.5 times higher than HAR and 1.4 times higher than CHR hamsters, but the cholesterol gallstone incidence was practically equal for BIO and CHR hamsters (58% *vs.* 64% for the combined diets) and lowest for HAR hamsters (23%). When the same strains of hamsters were maintained on a purified cholesterol-free diet, CHR hamsters revealed significantly higher mol% biliary cholesterol and phospholipids and the lowest mol% bile acids with slightly supersaturated bile (LI of 1.0 ± 0.2) suggesting the onset of biliary cholesterol saturation even without dietary cholesterol (18). Thus, the LI *per se* is not necessarily a good predictor of cholesterol gallstone incidence. Factors such as antinucleating proteins (38), mucus production (39) or nucleation time presumably differ between hamster strains.

Lipoproteins and lithogenesis. Although all three strains of hamsters developed hyperlipemia in response to both levels of dietary cholesterol (0.4% *vs.* 0.8%), BIO and HAR hamsters had a markedly higher cholesterolemic and triglyceridemic response to the cholesterol-enriched diets than CHR hamsters. A similar sensitivity of the BIO hamster to dietary cholesterol and saturated fat was reported previously (16). Strain differences in the cholesterol lipoprotein distribution between CHR, BIO and HAR hamsters revealed that VLDL and IDL cholesterol were greatly expanded at the expense of HDL cholesterol (7,8). In the absence of dietary cholesterol the VLDL + LDL/HDL-cholesterol ratio was below 1.0 in all three strains (HAR, 0.4; CHR, 0.5; BIO, 0.8) (18), whereas this ratio increased after cholesterol feeding to 2.6 in BIO hamsters and to 1.5 and 1.6 in CHR and HAR hamsters, respectively.

Bile acid profile and gallstones. In the absence of dietary cholesterol, total cholate accounted for 50% of the total bile acids in CHR and 60% in BIO and HAR hamsters (18). Adding dietary cholesterol (this study) depressed the percent cholate to a different extent in the three hamster strains. Consequently, total cheno, normally representing 20% of the total bile acids in HAR, 30% in BIO and 46% in CHR hamsters, increased in all three hamster strains. In fact, cholesterol-fed CHR hamsters with the highest incidence of cholesterol gallstones demonstrated the highest percentage of cheno (61%) and the lowest percentage of cholate (31%), and the lowest cholate/cheno ratio (0.5), consistent with our previous results under the same dietary conditions (37). The shift in the bile acid profile was less pronounced in HAR hamsters, the only hamsters

with a cholate/cheno ratio that remained above 1.0 and which developed the least cholesterol gallstones. HAR hamsters fed a cholesterol-free diet maintained a cholate/cheno ratio of 3.3 (18).

The decline in the cholate/cheno ratio has been described previously under a variety of gallstone-inducing dietary conditions in hamsters (6,37,40,41). However, the striking strain differences in the cholate/cheno ratio and susceptibility to cholesterol gallstones emphasize the probable genetic influence on the relationship. Studies in rats (42) and hamsters (43) have led others to suggest that dietary cholesterol depresses 12α-hydroxylase activity, the key enzyme in cholate synthesis. However, extensive hepatic cholesterol accumulation does not necessarily depress cholate in hamster bile (37). In fact, HAR hamsters herein had the greatest cholate profile despite having the greatest hepatic cholesterol accumulation, providing further evidence that cholesterol feeding and liver cholesterol *per se* are not causally related to depressed 12-OHase activity. Differences in lipoprotein and bile acid distribution observed in the three hamster strains in this study support the hypothesis (19) that circulating lipoprotein cholesterol precursor pools, rather than hepatic cholesterol, exert the primary modulating effect over the bile acid profile.

Compared to a similar diet without cholesterol (18), the cholesterol content of the present diet increased the cheno pool, and taurochenodeoxycholic acid was the predominant bile acid. The glycine/taurine ratio did not differ between the three strains, and bile acids were equally conjugated with both amino acids. The cholesterol challenge decreased the glycine/taurine ratio in all three strains although to a different extent. HAR hamsters demonstrated the biggest shift in the conjugation pattern (from 1.1 to 0.3) whereas the change was less pronounced in CHR hamsters (from 1.0 to 0.7).

Our data suggest that the hydrophilic/hydrophobic balance between glycine and taurine-conjugated tri- and dihydroxy bile acids, calculated as the HI (32), was the best predictor of cholesterol gallstones. Usually, hamsters fed chow or a cholesterol-free purified diet have an HI between +0.22 and +0.27 (18,32,37), but this index has been depressed as low as +0.14 by cholestyramine, which totally prevented cholesterol gallstone formation (37). The HI increased in all our hamster strains during cholesterol feeding in parallel with gallstone induction. Thus, HAR hamsters with a high-normal HI (+0.27) had the lowest gallstone incidence, whereas the large number of cholesterol gallstones in CHR hamsters was associated with a 1.5-fold increase in HI to +0.37. BIO hamsters were intermediate, both for HI (+0.34) and cholesterol gallstones. Taken together, the results suggest a strong relationship between the cheno/cholate ratio, HI and cholesterol gallstone formation. It is noteworthy that the bile acid contributing most to the HI, *i.e.*, deoxycholic acid, has been associated with the pathogenesis of gallstones in humans (44).

In summary, differences in lipoprotein and biliary lipid composition, including the LI and HI, and cholesterol gallstone formation were observed during dietary cholesterol challenge of three strains of hamsters. Comparison of these parameters suggests that CHR hamsters are more prone to cholelithiasis under these dietary conditions than the two other strains examined, but a clear link between specific lipoproteins and gallstones was not obvious. Thus, the source (environmental and nutritional) and genetic background of the animals are important variables when cholesterol metabolism and cholelithiasis are evaluated in this species.

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Hydrolysis of Fish Oils Containing Polymers of Triacylglycerols by Pancreatic Lipase *in vitro*

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Fish oils containing different levels of polymers of triacylglycerols formed during autoxidation were incubated with pancreatic lipase to establish whether these polymers are substrates for lipase hydrolysis. With oils containing low amounts (less than 4%) of triacylglycerol polymers as substrates, both triacylglycerols and polymers of triacylglycerols were almost completely hydrolyzed, and fatty acid monomers and monoacylglycerols were the major lipid products. Under the same incubation conditions, some triacylglycerols remained intact when highly oxidized oils containing 20 or 30% triacylglycerol polymers were the substrate. The fatty acid composition of these residual triacylglycerols was almost identical to that of triacylglycerols present at the start of the assay. When fish oil containing 30% triacylglycerol polymers was incubated with the lipase, the component triacylglycerols and polymers of triacylglycerols were hydrolyzed at similar rates, and fatty acid dimers were detected as a product. It is concluded that the high molecular weight polymers of triacylglycerols present in oxidized fish oils can be hydrolyzed by pancreatic lipase *in vitro*.

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There is currently much interest in fish oils in relation to human health, largely on account of their high content of n-3 polyunsaturated fatty acids (PUFA) (for reviews, see ref. 1). Although many studies have examined the effects of dietary fish oils on the lipid composition of blood and tissues in mammals (1–3), far less attention has been directed toward the initial digestion and absorption of the oils.

As with fats in general, the triacylglycerols which comprise the bulk of fish oils are hydrolyzed by pancreatic lipase in the intestinal lumen to free fatty acids and 2-monacylglycerols which are then absorbed by the enterocytes of the intestinal wall (4). Evidence from *in vitro* (5–7) and *in vivo* (8,9) studies suggests that long-chain PUFA characteristics of fish oils are more slowly liberated from triacylglycerol molecules than shorter chain, less unsaturated fatty acids. Possible bases for the discrimination of the digestive lipases against PUFA include chain length, degree of unsaturation and their distribution between the primary and secondary positions of the triacylglycerol molecule. Peroxidized PUFA, however, do not appear to be discriminated against during the hydrolysis of triacylglycerols by pancreatic lipase (10).

Commercial fish oils have recently been shown to contain small amounts of high molecular weight polymer material (11,12). These polymers most likely arise from the intermolecular linking of triacylglycerol molecules *via* peroxy bridges or other linkages formed during the autoxidation of their component PUFA. Studies on the enzymatic hydrolysis of thermally oxidized plant oils have shown that high molecular weight polymers of triacylglycerols are poor substrates for pancreatic lipase *in vitro* (13,14). No information, however, is available on the extent to which the polymers of triacylglycerols in oxidized fish oils can act as substrates for mammalian digestive lipases. In the present study, fish oils containing such polymers were subjected to hydrolysis with pancreatic lipase *in vitro* to establish whether these high molecular weight polymers are potential substrates for this enzyme *in vivo*.

MATERIALS AND METHODS

Fish oils. Cod liver oil (CLO) was obtained from Peter Moller A/S (Oslo, Norway). Chromatographically purified lipid (CPL) was the Fish Oil 30 manufactured by CPL Company AB (Karlshamn, Sweden). The oils contained no added antioxidant. To promote oxidation, 40 g samples of both oils were stirred for either 4 or 8 d in open beakers of 4.8 cm internal diameter at 35°C under irradiation from an artificial daylight fluorescent tube (1.1×1019 $Qm^{-2}s^{-1}$). CLOs subjected to these conditions for 4 and 8 d were designated CLO-4 and CLO-8, respectively. The corresponding CPL oils were refered to as CPL-4 and CPL-8. Peroxide values were measured by iodometric titration (15), and thiobarbituric acid values were determined as described by Ke and Woyewoda (16). Anisidine and iodine values were measured according to IUPAC 2.504 (17) and Wij's method (18), respectively. Polymer content was determined by size exclusion high-performance liquid chromatography (HPLC) as described previously (11).

Lipase hydrolysis. Purified porcine pancreatic lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) were purchased from Sigma Chemical Co. Ltd. (Poole, United Kingdom) and was approximately 75% protein with 20,000 to 50,000 units per mg protein. Colipase purified from porcine pancreas was also obtained from Sigma. The standard assay system employed comprised $CaCl_2$ (0.18% wt/vol), sodium deoxycholate (0.01% wt/vol), lipase and fish oil (2% vol/vol) in 0.5 M Tris/HCl, pH 7.7 and total volume 5.0 mL. Each assay contained 10,000 units of purified lipase and 10 μg colipase. Assays were carried out in sealed tubes with stirring at 37°C in the dark. When required, aliquots were removed from incubations for lipid analysis.

Lipid analysis. Total lipid was extracted from whole assays or aliquots with chloroform/methanol (2:1, vol/vol) (19). The analysis of lipid extracts by size exclusoin HPLC was carried out as previously described using three columns connected in series (20). Values quoted are percentage of total area obtained for each analysis. When required, lipid class composition was also determined by high-performance thin-layer chromatography (HPTLC) using hexane/diethyl ether/glacial acetic acid (80:20:2, by

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Abbreviations: CLO, cod liver oil; CPL, chromatographically purified lipid; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; UV, ultraviolet.

vol) as the developing solvent system. The copper acetate reagent of Fewster *et al.* (21) was used to visualize the separated components which were subsequently quantitated using a Shimadzu CS-930 dual wavelength thin-layer chromatography (TLC) scanner linked to a Shimadzu DR-2 data recorder (Tokyo, Japan). For confirmation of the identity of diacylglycerols, HPTLC plates were impregnated with boric acid by immersion in 1.2% (wt/vol) boric acid in ethanol/water (1:1, vol/vol) and dried at 105°C for 5 min. Samples and standards were then applied, and the plates developed in chloroform/acetone (96:4, vol/vol). Developed chromatograms were visualized as above.

To establish whether components containing intermolecular peroxy (C–OO–C) linkages were present, lipid recovered from lipase incubations with highly oxidized fish oil were treated with stannous chloride (22). Prior to its use for this purpose, the method was evaluated by applying it to methyl esters of cod roe total lipid, which had been oxidized under ultraviolet (UV) light for two days to produce polymers. Saponification of lipase products was performed using 1M KOH in 95% ethanol (19).

For fatty acid analysis, lipid classes were separated by TLC using 20 × 20 cm glass plates coated with silica gel G and the solvent system described above for HPTLC densitometry. The separated lipid classes were transesterified directly on the adsorbent with 1% H_2SO_4 in methanol to prepare methyl esters of the fatty acids (19). Fatty acid methyl esters were analyzed by capillary gas-liquid chromatography (GLC) using a 50 m × 0.32 mm i.d. CP Wax 51 fused silica column and on-column injection under conditions previously described (23).

RESULTS

Prior to the exposure of oils to oxidizing conditions, the initial peroxide, anisidine and thiobarbituric acid values of the cod liver oil (CLO-0) were all higher than those of the chromatographically purified fish oil, CPL-0 (Table 1). The triacylglycerol polymer content of the CLO-0 (1.6%) was also slightly higher than that of CPL-0 (1.3%). Conversely, the iodine value of CLO-0 (169) was lower than that of CPL-0 (222). The characteristics of both oils were changed markedly by exposure to oxidizing conditions. Peroxide, anisidine and thiobarbituric acid values and triacylglycerol polymer content were all related to the length of time exposed to the oxidizing conditions. The anisidine value of CLO-8 was double that of CPL-8, whereas the differences between these two oils in terms of peroxide and thiobarbituric acid values were not so great. Although little difference in iodine value was observed between starting oils and those oxidized for 4 d, the iodine values of CLO-8 and CPL-8 were substantially less than those of CLO-0 and CPL-0, respectively. The triacylglycerol polymer contents of CLO-4 and CPL-4 were more than double those of CLO-0 and CPL-0, respectively, as determined by HPLC analysis. In CLO-8, 30.3% of the oil was polymer material compared to only 20.4% in CPL-8. The polymers present in CLO-0 and CLO-4 consisted of only dimers of triacylglycerols whereas the polymers in CLO-8 were made up of 60% dimers, 24% trimers and 16% more highly polymerized forms of triacylglycerols. Similarly, only dimers of triacylglycerols were present in the CPL-0 and CPL-4 oils, but the polymer fraction of CPL-8 comprised 80% dimers, 18% trimers and 2% higher polymers.

TABLE 1

Characteristics of Fish Oils[a]

	PV (meq/kg)	AV	TBA (μmol/g)	IV	TAG Polymer (%)
CLO-0	4.3	23	0.7	169	1.6
CLO-4	82.0	38	5.4	169	3.7
CLO-8	940.0	360	39.0	139	30.3
CPL-0	3.0	2	0.3	222	1.3
CPL-4	130.0	20	6.6	220	3.4
CPL-8	740.0	180	33.0	206	20.4

[a]Abbreviations used are: CLO, cod liver oil; CPL, chromatographically purified lipid; PV, peroxide value; AV, anisidine value; TBA, thiobarbituric acid; IV, iodine value; TAG, triacylglycerol.

Analysis of the starting oils by HPLC showed that triacylglycerols were the major component of all oils (Table 2). A small amount of cholesterol was present in the cod liver oils but was absent from the CPL oils. Preliminary time course experiments in which HPTLC was used to follow the disappearance of triacylglycerols from CLO-0 and CPL-0 established that under the assay conditions employed triacylglycerols were almost entirely hydrolyzed by the lipase in 1 h. This incubation time was used in subsequent assays of lipase action on all oils.

The composition, as determined by HPLC, of total lipid extracted from assays after incubation of the oils with the purified pancreatic lipase is presented in Table 2. Polymers and dimers of fatty acids, if present, co-elute with triacylglycerols and diacylglycerols, respectively (14), when analyzed by size exclusion HPLC under conditions similar to those employed in this study. In all assays with CLO as substrate, monoacylglycerols and fatty acid monomers were the major lipid classes recovered at the end of the incubation period. Fatty acid monomers accounted for some 50% of the lipid from assays with CLO-0 and CLO-4 but only 34% of that from CLO-8. Monoacylglycerols were the second most abundant lipid class extracted from all assays with the exception of the CLO-8 assay in which the levels of fatty acid monomers and monoacylglycerols were equal. Higher proportions of components corresponding to triacylglycerols plus fatty acid polymers and diacylglycerols plus fatty acid dimers were present in the assays with CLO-8 than with CLO-4 or CLO-0. No polymers of triacylglycerols were recovered in the lipid extracted from assays with CLO-0 and CLO-4, but very small amounts were observed in the assays conducted with CLO-8.

The pattern observed with CPL oils as substrates (Table 2) was generally similar to that obtained with CLO oils. For CPL-0 and CPL-4, fatty acid monomers were the major lipid class recovered from each assay followed by monoacylglycerols. However, with CPL-8 as substrate, the proportion of fatty acid monomers present in the lipid extracted from assays was notably less than was observed when CPL-0 and CPL-4 were the substrate oils. The level of triacylglycerols plus fatty acid polymers recovered from assays with CPL-8 was higher than that observed with the other two oils. No polymers of triacylglycerols were recovered from assays with CPL-0 and CPL-4, whereas

TABLE 2

Lipid Class Composition (% total lipid) as Determined by HPLC Analysis of Oils Before and After Incubation with Pancreatic Lipase[a]

	CLO-0	CLO-4	CLO-8
Initial composition			
Triacylglycerol			
polymers	1.6 ± 0.1	3.7 ± 0.9	30.3 ± 0.8
monomers	96.7 ± 0.2	94.9 ± 1.0	69.0 ± 0.8
Cholesterol	1.7 ± 0.2	1.4 ± 0.2	0.7 ± 0.0
Final composition			
Triacylglycerol			
polymers	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4
monomers + FAP	6.6 ± 3.6	1.8 ± 1.0	11.6 ± 7.9
Diacylglycerols + FAD	9.4 ± 2.6	7.8 ± 2.0	19.5 ± 8.1
Monoacylglycerols	33.9 ± 4.5	40.5 ± 4.1	34.4 ± 7.1
Cholesterol	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
Fatty acid monomers	49.7 ± 2.4	49.8 ± 1.7	33.9 ± 6.6
	CPL-0	CPL-4	CPL-8
Initial composition			
Triacylglycerol			
polymers	1.3 ± 0.1	3.4 ± 0.1	20.4 ± 0.7
monomers	98.7 ± 0.0	96.6 ± 0.1	79.6 ± 0.7
Final composition			
Triacylglycerol			
polymers	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.0
monomers + FAP	6.2 ± 3.3	1.7 ± 1.2	17.6 ± 2.8
Diacylglycerols + FAD	10.5 ± 4.0	7.9 ± 2.1	18.4 ± 4.9
Monoacylglycerols	34.4 ± 4.3	38.4 ± 6.1	26.0 ± 3.4
Cholesterol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fatty acid monomers	48.5 ± 2.3	51.9 ± 3.4	37.8 ± 4.2

[a]Values are means ± SD of three analyses or incubations. Abbreviations used are: CLO, cod liver oil; CPL, chromatographically purified lipid; FAP, fatty acid polymers; FAD, fatty acid dimers; HPLC, high-performance liquid chromatography.

TABLE 3

Effects of Saponification and Stannous Chloride ($SnCl_2$) Reduction on the Composition of the Products of Lipase Hydrolysis of CLO-8[a]

	Treatment			
Lipid class	None	Saponification	$SnCl_2$ reduction	$SnCl_2$ reduction + saponification
Triacylglycerol polymers	0.4	—	0.3	—
Triacylglycerol monomers	11.6	—	11.8	—
Diacylglycerols	19.5	—	14.6	—
Dimer fatty acids		3.3		4.0
Monoacylglycerols	34.4	—	35.0	—
Fatty acid monomers	33.9	95.8	38.0	95.0
Cholesterol	0.2	0.9	0.3	1.0

[a]Values are means of two experiments. CLO, cod liver oil.

some 1% of the lipid extracted from assays with CPL-8 was triacylglycerol polymeric material. The component corresponding to diacylglycerols plus fatty acid dimers was also a major lipid product of assays with CPL-8. Qualitative HPTLC analysis of the lipid extracted from assays generally agreed with the results of the HPLC analysis and, in the case of assays with CLO-8 and CPL-8 oils, showed the presence of a small amount of polar material which remained mostly at the origin but also ran as a diffuse band to just above the monoacylglycerols.

To establish whether polymer and dimer fatty acids were present as products of lipase hydrolysis, the lipids extracted from 1-h incubations of CLO-8 with lipase were subjected to saponification or stannous chloride reduction and analyzed by HPLC. In addition, samples which had been treated with stannous chloride were subsequently saponfied and re-analyzed. Of the saponified lipolysis products, 95.8% was monomeric fatty acids and 3.3% was fatty acid dimers which had the same retention time as the diacylglycerols in the original lipase products (Table 3).

Treatment of the lipolysis products with stannous chloride resulted in a decrease in the proportion of the component with retention time equal to that of diacylglycerols from 19.5 to 14.6%. At the same time the proportion of fatty acid monomers increased from 33.9 to 38.0%. Saponification of the CLO-8 after lipolysis and treatment with stannous chloride yielded 4% fatty acid dimers, the remainder being almost exclusively monomer fatty acids with 1% cholesterol. No fatty acid polymers were observed after any treatment.

The summarized fatty acid classes isolated by TLC from assays with the original oils and oils which had been oxidized for 8 d are compared in Tables 4 and 5 with those of the triacylglycerols present in these oils at the start of the assays. The triacylglycerols originally present in CLO-0 contained some 30% PUFA whereas only 18.8% of the fatty acids in CLO-8 triacylglycerols were polyunsaturated (Table 4). The ratio of 22:6n-3 to 20:5n-3 in CLO-0 triacylglycerols was 1.3 but only 1.1 in those of CLO-8. Residual triacylglycerols isolated from lipase incubations with CLO-8 were similar in fatty acid composition to those originally present before the initiation of lipolysis. PUFA accounted for 46.4% of fatty acids in CPL-0 triacylglycerols but only 38.7% in those of CPL-8 (Table 5). The ratios of 22:6n-3 to 20:5n-3 in CPL-0 and CPL-8 triacylglycerols were 0.9 and 0.7, respectively, and the fatty acid composition of the triacylglycerols remaining in assays with CPL-8 at the end of the incubation period was almost identical to that present at the start of the lipase digestion. With all substrate oils examined,

TABLE 4

Summarized Fatty Acid Compositon (wt%) of Original Triacylglycerols and Products of Lipolysis of Cod Liver Oils[a]

Fatty acids	Initial TAG	Final			
		TAG	DAG	MAG	FFA
CLO-0					
Total saturated	16.5	—	16.1 ± 0.7	22.1 ± 1.7	13.5 ± 1.1
Total monoenoic	53.6	—	38.7 ± 3.1	35.0 ± 0.9	67.7 ± 2.7
20:5n-3	8.3	—	17.3 ± 2.0	11.5 ± 0.4	6.1 ± 1.0
22:6n-3	10.4	—	16.8 ± 0.9	21.9 ± 1.6	4.4 ± 0.7
Total PUFA	29.9	—	44.9 ± 3.2	42.7 ± 2.3	18.7 ± 2.0
22:6n-3/20:5n-3	1.3	—	1.0 ± 0.1	1.9 ± 0.1	0.7 ± 0.1
CLO-8					
Total saturated	19.8	18.9 ± 0.3	23.2 ± 0.8	29.2 ± 0.7	17.6 ± 0.4
Total monoenoic	61.0	61.9 ± 0.4	51.0 ± 4.1	48.7 ± 2.7	75.4 ± 0.2
20:5n-3	5.3	5.7 ± 0.3	9.6 ± 2.2	5.9 ± 0.6	1.3 ± 0.1
22:6n-3	5.9	6.2 ± 0.4	7.7 ± 1.4	8.8 ± 1.4	0.9 ± 0.1
Total PUFA	18.8	19.0 ± 0.6	25.2 ± 4.4	21.8 ± 2.5	6.9 ± 0.6
22:6n-3/20:5n-3	1.1	1.1 ± 0.0	0.8 ± 0.1	1.5 ± 0.1	0.7 ± 0.1

[a]Values are means ± SD of three assays. Abbreviations used are: CLO, cod liver oil; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; PUFA, polyunsaturated fatty acids.

TABLE 5

Summarized Fatty Acid Compositon (wt%) of Original Triacylglycerols and Products of Lipolysis of Chromatographically Purified Oils[a]

Fatty acids	Initial TAG	Final			
		TAG	DAG	MAG	FFA
CLO-0					
Total saturated	27.2	—	18.3 ± 0.9	26.2 ± 1.0	26.7 ± 0.3
Total monoenoic	26.0	—	14.1 ± 1.8	15.2 ± 1.0	33.1 ± 0.8
20:5n-3	15.3	—	31.9 ± 4.2	15.7 ± 0.7	17.3 ± 0.2
22:6n-3	13.9	—	18.2 ± 0.7	26.2 ± 0.3	7.9 ± 0.4
Total PUFA	46.4	—	67.5 ± 2.7	58.6 ± 0.8	40.2 ± 1.4
22:6n-3/20:5n-3	0.9	—	0.6 ± 0.1	1.7 ± 0.1	0.5 ± 0.1
CPL-8					
Total saturated	30.8	30.5 ± 0.7	31.9 ± 2.5	37.5 ± 2.7	37.9 ± 0.8
Total monoenoic	30.3	28.7 ± 1.3	24.5 ± 2.5	24.0 ± 1.3	47.2 ± 0.8
20:5n-3	14.9	16.4 ± 1.5	19.3 ± 3.6	11.8 ± 0.7	4.8 ± 0.4
22:6n-3	10.3	10.1 ± 0.7	10.3 ± 1.2	12.6 ± 1.9	2.0 ± 0.1
Total PUFA	38.7	39.9 ± 2.4	43.4 ± 4.9	38.3 ± 3.8	14.8 ± 0.5
22:6n-3/20:5n-3	0.7	0.6 ± 0.0	0.5 ± 0.1	1.1 ± 0.1	0.5 ± 0.0

[a]Footnote as in Table 3. CPL, chromatographically purified lipid.

the free fatty acid fraction after lipase hydrolysis contained a higher proportion of monenoic fatty acids than the starting triacylglycerols or partial acylglycerols. This was particularly true of assays with CLO-0 and CLO-8 whose triacylglycerols were initially richer in monoenoic acids than the equivalent CPL oils. Of the free fatty acids recovered from the assays with CLO-0 and CLO-8, 68 and 75%, respectively, were monounsaturated. With CLO-0 as substrate the content of PUFA in diacylglycerols and monoacylglycerols were similar and both higher than that of the starting triacylglycerols. However, it was notable that whereas the levels of 20:5n-3 and 22:6n-3 were similar at around 17% in the diacylglycerols, the level of 22:6n-3 in monoacylglycerols (22%) was almost twice that of 20:5n-3. With CLO-8 oil also, the diacylglycerols and monoacylglycerols were of similar PUFA content, and again the ratio of 22:6n-3 to 20:5n-3 in monoacylglycerols was almost double that in diacylglycerols. Similarly, with CPL-0 and CPL-8 as substrates, the ratio of 22:6n-3 to 20:5n-3 in monoacylglycerols was far greater than that of the starting triacylglycerols or the diacylglycerol and free fatty acid products.

To compare the rates of hydrolysis of triacylglycerol monomers and polymers, CLO-0 and CLO-8 were incubated with purified lipase and the lipid classes present in the assay examined by HPLC with respect to time (Fig. 1). With CLO-0 as substrate, the proportion of triacylglycerol monomers decreased rapidly over the initial 15 min and was balanced by increases in those of fatty acid monomers, monoacylglycerols and diacylglycerols plus fatty acid dimers. After 60 min, only 2% of the lipid present in the assay was triacylglycerol monomers, and fatty acid monomers closely followed by monoacylglycerols were the predominant lipids. Apart from the zero time point, polymers of triacylglycerols were not observed in samples taken at various times from the incubation.

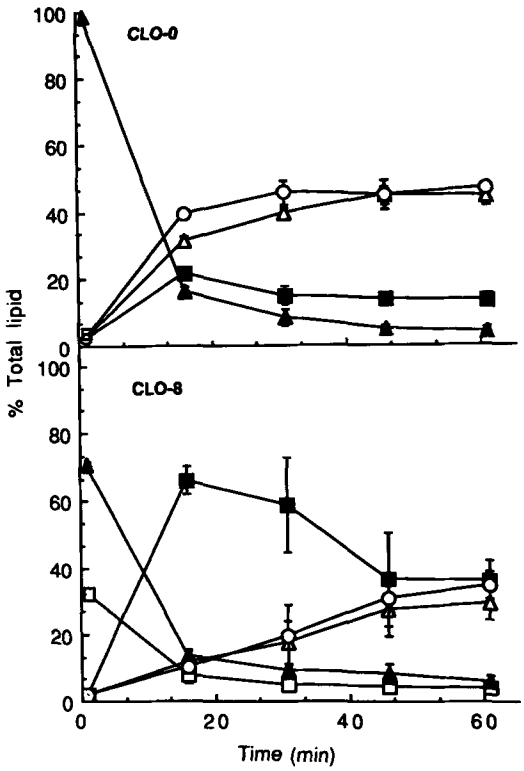

FIG. 1. Time course of hydrolysis of CLO-0 and CLO-8 with purified pancreatic lipase. Experimental conditions were as described in the text. Vertical bars represent SD obtained with three incubations. □—□, triacylglycerol polymers; ▲—▲, triacylglycerol monomers; ■—■, diacylglycerols + fatty acid dimers; △—△, monoacylglycerols; ○—○, fatty acid monomers. CLO, cod liver oil.

A different pattern of products was observed when CLO-8 was employed as substrate (Fig. 1). The proportions of triacylglycerol monomers and polymers in this oil decreased rapidly during the first 15 min. During this time the proportion of the component corresponding to diacylglycerols plus dimer fatty acids increased to 65% of the total lipid and decreased thereafter to a final value of 34% observed after 60 min. The levels of fatty acid monomers and monoacylglycerols increased almost equally with time of exposure of the oil to the lipase. The proportions of cholesterol remained constant at around 1.2 and 0.6% in assays with CLO-0 and CLO-8, respectively (data not shown). When the lipid extracted from the incubations with CLO-8 was analyzed by qualitative HPTLC, time-dependent changes similar to those observed with HPLC were discernable in the levels of triacylglycerols and free fatty acids. Apart from an initial increase over the first 15 min, no changes in the proportions of diacyl- and monoacylglycerols were obvious, and polar material was observed streaked above the origin at all time points. Analysis of lipid extracts using HPTLC plates impregnated with boric acid showed the diacylglycerols were always predominantly the 1,2-diacylglycerols. When the HPLC data obtained for triacylglycerols and triacylglycerol polymers were expressed as a percentage of their initial values in CLO-8, it was notable that these two lipid classes were hydrolyzed at similar rates. Thus, relative to their initial starting values, 80.9 and 83.2% of the triacylglycerol polymers and monomers, respectively, were hydrolyzed over the initial 15 min. The rate of hydrolysis over the subsequent 45 min was much less for each.

DISCUSSION

As found previously (11), the amount of polymer material of high molecular weight present in fish oils was directly related to the length of time the oils were exposed to conditions favoring the autoxidation of PUFA. A notable feature was that the formation of polymers in the CPL was less extensive than in the conventional CLO. This may be attributed to the virtual absence in the CPL (manufacturer's data sheet) of metal ions which can act as prooxidants.

The present results conform to the well established fact that fish oil triacylglycerols are efficiently hydrolyzed by mammalian pancreatic lipase *in vitro* (24). It was notable that monoacylglycerols accounted for around 33% of the final lipid product in incubations with original oils and oils which had been oxidized for 4 d. Since these oils originally comprised predominantly triacylglycerol monomers, this value is consistent with the purified lipase specifically hydrolyzing the fatty acids from the 1- and 3-position but not utilizing the resultant 2-monoacylglycerol as a substrate. This is in keeping with the known substrate specificity of the lipase (24).

The very high content of monoenoic acids in the free fatty acid fraction of all lipase-digested oils is consistent

with the lipase hydrolyzing the primary positions of the triacylglycerol molecules since monoenoic fatty acids are known to be preferentially esterified in position *sn*-3 of CLO triacylglycerols (25). Likewise, the high proportions of 22:6n-3 in monoacylglycerols suggest that, in keeping with its known stereospecific distribution in CLO triacylglycerols (25), this PUFA was concentrated in the *sn*-2 position of the original triacylglycerol molecules.

The lower ratio of 22:6n-3 to 20:5n-3 in the triacylglycerols of the heavily oxidized oils than in those of the original oils suggests that within triacylglycerols 22:6n-3 is more prone to autoxidation than 20:5n-3. The autoxidation of methyl ester derivative of 22:6n-3 is also known to be more extensive than that of 20:5n-3 (26). Given that 22:6n-3 is heavily oxidized during autoxidation of CLO, it might be expected that the position *sn*-2 of triacylglycerols will contain a higher proportion of autoxidized fatty acids than positions *sn*-1 and *sn*-3 simply on account of the high level of 22:6n-3 located in that position. It has been shown recently, however, that the oxidation of trilinoleoyl- and trilinolenoylglycerol is not selective toward either the 1(3)- or 2-position (27,28). A previous comparison of triacylglycerols in fresh sardine oil with that displaying moderate autoxidation as a result of freeze-thawing found no difference in the molecular species composition of the triacylglycerol present suggesting that autoxidation in fish oils is also a random process (29). Nevertheless, the highly unsaturated nature of the 2-position of fish oil triacylglycerols suggests that this position may be heavily involved in the formation of polymers of triacylglycerols by intermolecular condensation of peroxy radicals. The relative importance of PUFA situated in the different positions of triacylglycerols in intermolecular linking remains to be examined.

A recent study of the specificity of hydrolysis of triacylglycerols in menhaden oil by pancreatic lipase *in vitro* showed clearly that the triacylglycerols remaining after 70% hydrolysis of the oil were enriched in C_{20} and C_{22} fatty acids and that PUFAs were most resistant to hydrolysis (7). Although not studied in detail, no evidence for this specificity with fish oils was observed in the present study since residual triacylglycerols were almost identical to the original triacylglycerols in fatty acid composition. It was notable, however, that residual, nonhydrolyzed triacylglycerols were only present in assays with highly oxidized oils. Although this may imply that the presence of an autoxidized fatty acid in a triacylglycerol molecule slows its rate of hydrolysis by pancreatic lipase, it does not agree with a recent suggestion that triacylglycerols containing hydroperoxy linoleoyl and hydroperoxy linolenoyl groups are more hydrophilic in nature and may be more susceptible to hydrolysis by lipase (10). Nothing is known of the selectivity of lipase toward the peroxidized longer chain PUFA which will be present in highly oxidized fish oils. It is possible that the triacylglycerol monomers which remain after the incubation of highly-oxidized fish oils with lipase contain intramolecular linkages between component PUFA. Such linkages can be expected to be resistant to lipase hydrolysis and thus hinder the breakdown of the triacylglycerol. However, since any cross-linked fatty acids would not be detected by the procedure used for the comparison of relative fatty acid compositions, the present study does not provide definite evidence for the above situation.

Although the rates of hydrolysis of isolated triacylglycerol monomers and polymers were not compared in the present study, it was found that their rates of hydrolysis were very similar when presented simultaneously as components of a highly oxidized oil. This observation contrasts with the results of a similar previous study with thermally oxidized plant oils which showed that dimeric and polymeric acylglycerols were resistant to hydrolysis by pancreatic lipase *in vitro* (13). The authors of a more recent study in which triacylglycerol monomers and polymers isolated from heat-treated olive oil were exposed to pancreatic lipase *in vitro* also noted a slower rate of hydrolysis with the polymer fraction (14). It remains to be established whether similar differences in the rates of lipolysis exist between triacylglycerols monomers and polymers when they are isolated from autoxidized fish oils and presented separately to the lipase.

Peroxy bridges (C-OO-C) are known to be broken by reduction with stannous chloride, whereas carbon (C-C) and ether (C-O-C) linkages, as well as ester bonds, are resistant to this treatment (22). Alkaline hydrolysis, on the other hand, breaks both ester bonds and peroxy bridges but not C-C or C-O-C linkages (30). Consequently, the changes observed upon treatment of the CLO-8 lipolysis products with stannous chloride are consistent with the presence in the lipolysis products of peroxy-linked fatty acid dimers which co-elute with diacylglycerols under the HPLC conditions employed. The additional presence of fatty acid dimers containing ether or carbon linkages as products was revealed by saponification of the lipid products of lipase hydrolysis. Taken together, these observations provide evidence that the hydrolytic degradation of the triacylglycerol polymers of highly oxidized fish oils by pancreatic lipase gives rise to fatty acid dimers containing more than one type of linkage. A recent study has also demonstrated that fatty acid dimers are released from thermally oxidized olive oil when acted upon by pancreatic lipase *in vitro* (14).

With respect to the hydrolysis of triacylglycerol polymers by pancreatic lipase, extrapolation of the results obtained with thermally oxidized plant oils to air-oxidized fish oils is probably inaccurate although similarities are bound to exist. Apart from the obvious differences in fatty acid composition between plant oils and fish oils, the type of linkages present in triacylglycerol polymers may have an influence on the susceptibility of these compounds to lipase hydrolysis. Oxidation induced at the high temperatures used to produce thermally-oxidized oils is likely to generate a higher proportion of C-C linkages than autoxidation brought about by the passage of air or oxygen at moderate temperatures, a situation which favors the formation of peroxy linkages between PUFAs in their methyl ester form (31). Thus, the results of studies using thermally oxidized plant oils may differ considerably from those obtained with air-oxidized fish oils.

In summary, the present study shows that highly oxidized fish oils containing high levels of triacylglycerol polymers are hydrolyzed by pancreatic lipase *in vitro* less efficiently than fish oils which contain only moderate or low levels of these polymer compounds. Nevertheless, the high molecular weight polymers of oxidized fish oils can be degraded by lipase *in vitro* yielding fatty acid dimers as well as the normal products of triacylglycerol lipolysis. Further characterization of the nature of the

triacylglycerol polymers in autoxidized fish oils and the products of their lipolytic breakdown *in vitro* are merited before their digestion and absorption by mammals *in vivo* are considered.

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Stearidonic Acid, an Inhibitor of the 5-Lipoxygenase Pathway. A Comparison with Timnodonic and Dihomogammalinolenic Acid

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Leukotrienes have been shown to play an important role as mediators in various disease processes, including asthma and inflammation; thus, their synthesis is tightly regulated. The major precursor of leukotrienes is arachidonic acid (20:4n-6). Fatty acids which are structurally similar to 20:4n-6, such as eicosatrienoic acid (20:3n-6; dihomogammalinolenic acid) and eicosapentaenoic acid (20:5n-3; timnodonic acid) have been found to inhibit leukotriene biosynthesis. Because of the structural similarity of octadecatetraenoic acid (18:4n-3; stearidonic acid) with 20:4n-6, the present study was undertaken to determine whether stearidonic acid also exerts an inhibitory effect on the 5-lipoxygenase pathway. Human leukocytes were incubated with 18:4n-3 (20 μM or 10 μM), 20:5n-3 (20 μM) or 20:3n-6 (20 μM) and subsequently stimulated with 1 μM ionophore A23187 and 20:4n-6 (20 μM or 10 μM). The 5-lipoxygenase products were then measured by high-performance liquid chromatography. Leukotriene synthesis was reduced by 50% with 20 μM 18:4n-3 and by 35% with 10 μM 18:4n-3. Formation of 5S,12S-di-hydroxy-eicosatetraenoic acid and of 5-hydroxy-eicosatetraenoic acid was decreased by 25% with 20 μM 18:4n-3 and by 3% with 10 μM 18:4n-3. The inhibition observed with 20 μM 18:4n-3 appeared to be of the same order as that observed with 20 μM 20:5n-3; the inhibition observed with 18:4n-3 was shown to be dose-dependent. The inhibition produced by 20 μM 20:3n-6 was greater than that observed with either 20 μM 18:4n-3 or with 20 μM 20:5n-3. The results suggest that stearidonic acid, which is found, for example, in blackcurrant seed oil (which also contains the 20:3n-6 precursor), may play a role in suppressing inflammation.
Lipids 28, 321–324 (1993).

In stimulated human polymorphonuclear leukocytes (PMN), 5-lipoxygenase converts arachidonic acid to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE). The 5-HPETE is then reduced to 5-HETE by glutathione peroxidase or transformed to a variety of leukotrienes (1–5) (Fig. 1). Leukotriene B_4 (LTB_4) possesses potent biological activities and is involved in leukocyte adhesion, aggregation and degranulation (6–10). Thus, because of their involvement in leukotriene synthesis, leukocytes play an important role in inflammatory processes (5,11,12). Inhibition of 5-lipoxygenase and hence leukotriene synthesis could therefore be a way of down-regulating inflammatory events.

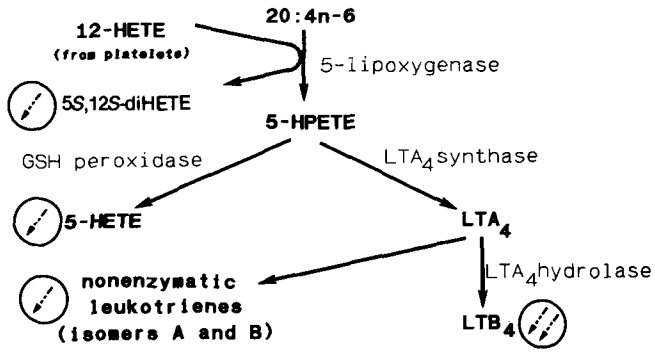

FIG. 1. Partial 5-lipoxygenase pathway in leukocytes. One circled slanted arrow indicates that less product was formed relative to the controls when the leukocytes were incubated with 18:4n-3, 20:5n-3 or 20:3n-6. Two circled slanted arrows indicate that much less product was formed. This suggests that the inhibition may have affected both 5-lipoxygenase and leukotriene A_4 (LTA_4) hydrolase. HETE, hydroxy-eicosapentaenoic acid; di-HETE, dihydroxylated eicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; LTB_4, leukotriene B_4.

There is increasing evidence that certain polyunsaturated fatty acids (PUFA) of the n-3 series present in fish oil can, by modulating leukotriene synthesis, favorably affect inflammatory conditions (13–17).

In the present study, the effects of stearidonic acid (18:4n-3), which occurs, for example, in blackcurrant seed oil (18), were tested in comparison with other PUFA, namely eicosapentaenoic acid (20:5n-3) and dihomogammalinolenic acid (20:3n-6). Isolated human leukocytes were incubated together with these fatty acids, and the 5-lipoxygenase end-products were separated by thin-layer chromatography (TLC) and quantified by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Reagents. Calcium ionophore A23187, dextran 250, eicosatrienoic acid (20:3n-6), arachidonic acid, (20:4n-6), eicosapentaenoic acid, (20:5n-3), prostaglandin B_2 and soybean lipoxygenase type 1 (EC 1.13.11.12.) were obtained from Sigma Chemical Company (St. Louis, MO). 5-HETE and LTB_4 were purchased from Cayman Chemicals Company (Ann Arbor, MI). 12-HETE was prepared from arachidonic acid (19). [1-^{14}C]Arachidonic acid (20:4n-6) was obtained from Dupont-NEN Products (Zürich, Switzerland). Stearidonic acid was isolated from blackcurrant seed oil and purified as described (20,21). [1-^{14}C]Stearidonic acid was prepared by total synthesis using the general procedures previously described (22). The Nucleosil C18, 3 μm column was obtained from Macherey-Nagel (Düren, Germany). Silica gel G plates and organic solvents (analytical grade) were purchased from Merck (Darmstadt, Germany). The 13-hydroxy derivative of 18:2 [13-hydroxy-octadecadienoic acid (HODE)] was prepared by soybean lipoxygenase treatment according to the method of Funk *et al.* (23).

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Abbreviations: di-HETE, dihydroxylated eicosatetraenoic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; HETE, hydroxy-eicosatetraenoic acid; HHT, hydroxy-heptadecatrienoic acid; HODE, hydroxy-octadecadienoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; PGB_2, prostaglandin B_2; PUFA, polyunsaturated fatty acid; R_t, retention time.

Preparation of cell suspensions. Human leukocytes were prepared according to our published procedure (19). Blood was collected into acid citrate dextrose as anticoagulant from normal donors who had not been under medication for at least 10 d prior to venipuncture. After centrifugation at 200 × *g* for 15 min, platelet-rich plasma was removed, and dextran 500 (0.5 vol of 3% in 0.9% NaCl) was added to the pellet. Sedimentation of the red cells was achieved in about 90 min at room temperature, and the upper leukocyte phase was centrifuged for 15 min at 80 × *g*. The sediment (about 3×10^6 cells) was resuspended into 1 mL of distilled water for 15 s, and 1 mL of double strength Tyrode *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) was added to restore the isoosmolarity of the leukocyte suspension. This suspension was centrifuged for 10 min at 60 × *g*, and the sedimented leukocytes were resuspended in Tyrode/HEPES containing 2 mM calcium chloride.

The leukocytes were incubated with either 10 μM or 20 μM 18:4n-3, 20 μM 20:5n-3, or 20 μM 20:3n-6 for 30 min at 37°C. They were then stimulated by 1 μM calcium ionophore A23187 and 20 μM 20:4n-6 for 10 min at 37°C. After acidification to pH 3 with 3N HCl, the lipids were extracted with cold ethanol/chloroform (3:6, vol/vol). Two nanomoles of 13-HODE and 2 nmol of prostaglandin B_2 (PGB_2) were added before extraction as internal standards to quantify the monohydroxylated fatty acids and the leukotrienes, respectively. The organic phase was immediately evaporated under nitrogen to avoid lactonization of the 5-hydroxy derivatives.

The 5-lipoxygenase response was also tested on 1 mL leukocytes (10^8 cells/mL) incubated with either 0, 10, 20, 40, 70 or 100 μM 18:4n-3 for 30 min at 37°C and were then stimulated by 1 μM ionophore A23187 and 10 μM 20:4n-6.

Analysis of monohydroxylated fatty acids. Lipid extracts were separated by two-dimensional TLC on silica gel G plates. In the first dimension, monohydroxylated fatty acids were separated with hexane/diethyl ether/acetic acid (60:40:1, by vol). The dihydroxylated fatty acids remained at the origin. The radiolabeled hydroxylated fatty acids were detected using a Berthold radioscanner, scraped off together with 13-HODE and extracted with diethyl ether, and the sample was then evaporated under nitrogen prior to analysis by HPLC. The hydroxy derivatives were then separated by reverse-phase HPLC, using a 4.6 mm by 250 mm column packed with Nucleosil C18 (3 μM). The eluent methanol/water (adjusted to pH 3 with acetic acid) (73:27, vol/vol) was pumped at a flow rate of 1 mL/min. The compounds were detected at λ = 234 nm and quantitated relative to the internal standard (2 nmol 13-HODE) assuming that all compounds had the same specific extinction ($\varepsilon = 3 \times 10^4$ M^{-1} cm^{-1}).

Analysis of dihydroxylated fatty acids. The dihydroxylated fatty acids which remained at the origin were eluted perpendicular to the first direction with hexane/diethyl ether/acetic acid (25:75:1, by vol). The dihydroxylated fatty acids were recovered by extraction with diethyl ether; the solvent was evaporated, and the metabolites were then separated by reverse-phase HPLC in the same way as described before except that the solvent was methanol/water (pH 3) (66:34, vol/vol). The internal standard PGB_2 was used to quantify the dihydroxylated fatty acids (λ = 270 nm, $\varepsilon = 2.8 \times 10^4$ and 5×10^4 M^{-1} cm^{-1} for PGB_2 and 5*S*,12*S* or *R*-dihydroxy derivatives, respectively) using a Hewlett-Packard diode array detector (model 1040A) (Basel, Switzerland).

Statistical analysis. Each data point represents the mean ± SEM of n determinations. The level of significance, P, was determined by a *t*-test against the corresponding control.

RESULTS

In the control group (Fig. 2), leukocytes were incubated with 1 μM ionophore A23187 and 20 μM arachidonic acid. The most prominent component observed was 5-HETE which had the same retention time (R_t = 42.69 min) as standard 5-HETE with a λ_{max} 234 nm. Hydroxyheptadecatrienoic acid (HHT) and 12-HETE was also detected by ultraviolet (UV) absorption (234 nm) at 15.42 and 36.14 min, respectively. HHT and 12-HETE are formed by platelet contamination. In this case the contamination was not more than 10 platelets per leukocyte.

In the presence of 18:4n-3 (Fig. 3), two compounds appeared with R_ts of 14.0 and 14.7 min, respectively. These components were also observed at a lower concentration, 10 μM of 18:4n-3, and were radioactive (results not shown). Their UV spectra (λ_{max} 234 nm) were similar to those

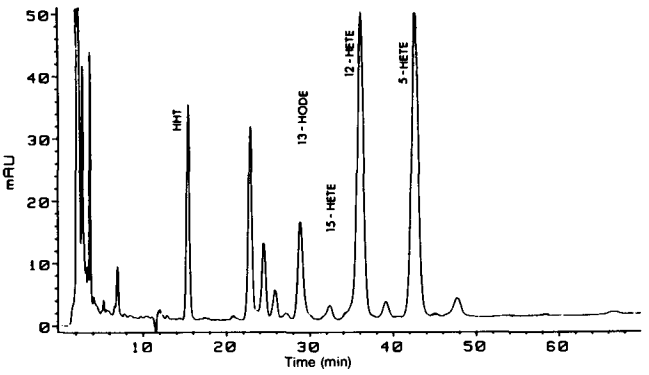

FIG. 2. Typical reverse-phase high-performance liquid chromatography profile of monohydroxylated fatty acids measured after triggering leukocytes with 20 μM arachidonic acid and 1 μM ionophore A23187. 13-hydroxy-octadecadienoic acid (HODE) was used as internal standard. Abbreviations as in Figure 1.

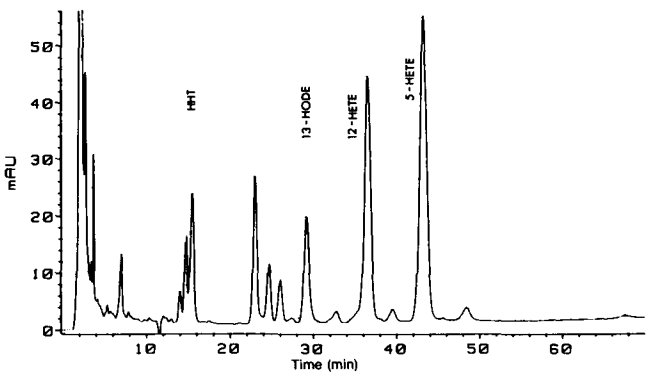

FIG. 3. Same profile as in Figure 2 but the leukocytes were preincubated for 30 min at 37°C with 20 μM stearidonic acid. See Figures 1 and 2 for abbreviations.

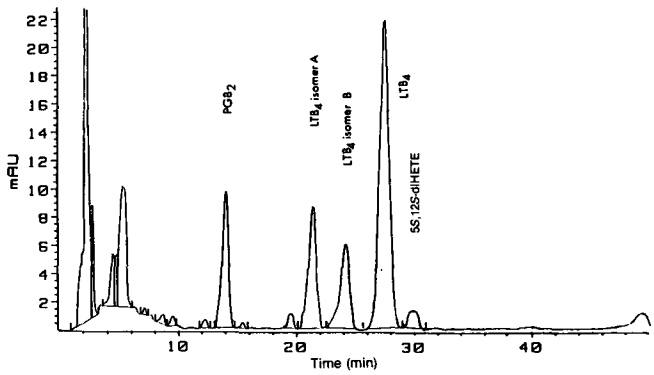

FIG. 4. Typical reverse-phase high-performance liquid chromatography (HPLC) profile of dihydroxylated fatty acids measured after stimulating leukocytes as described in Figure 2. Prostaglandin B_2 (PGB_2) was used as an internal standard. Abbreviations in Figure 1.

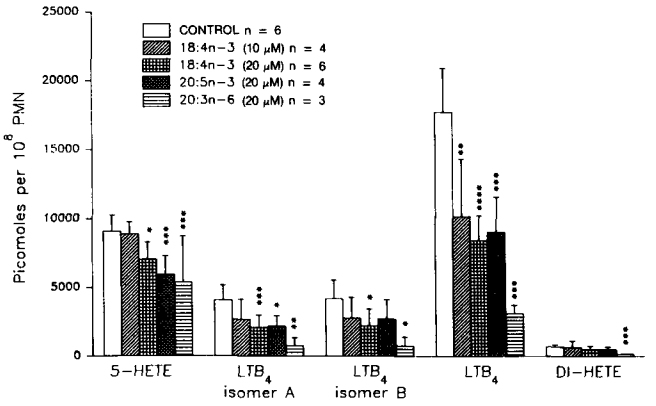

FIG. 5. Leukocytes (10^8 cells) were suspended in 1 mL of Tyrode *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) and incubated in the presence or absence of different fatty acids at the indicated concentrations for 30 min at 37°C. Afterward the cells were stimulated with 20 μM arachidonic acid and 1 μM ionophore A23187 for 10 min at 37°C. The 5-lipoxygenase products were quantified as described in Materials and Methods. The results are expressed in picomoles per 10^8 cells and represent means ± SEM of the five separate experiments with n determination within each. The levels of significance, *P*, were determined by a *t*-test: *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$, ****$P < 0.001$ when compared to the control. Abbreviations as in Figure 1.

obtained with standard monohydroxylated components but their levels were very low, with transformation of less than 2% of the radioactive substrate. Furthermore, the conversion of radiolabelled stearidonic acid to either 20:5n-3 or 22:6n-3 could not be detected in isolated leukocytes (results not shown).

The dihydroxylated fatty acids, isolated by TLC, were measured by HPLC using PGB_2 as internal standard (Fig. 4). Four main components were detected. The major metabolite was 5*S*,12*R*-[*Z*,*E*,*E*,*Z*]-LTB_4 since it had the same R_t(27.46 min) as did LTB_4. Its UV spectrum showed three λ_{max} at 261, 271 and 282 nm which is characteristic of compounds containing a conjugated triene. This compound was also radioactive when labelled arachidonic acid was the substrate. In addition two small more polar compounds with R_ts of 21.46 and 24.27 min, respectively, were detected, presumably corresponding to 5*S*,12*R*-[*E*,*E*,*E*,*Z*]-LTB_4 (LTB_4-isomer A) and 5*S*,12*S*-[*E*,*E*,*E*,*Z*]-LTB_4 (LTB_4-isomer B), as described previously

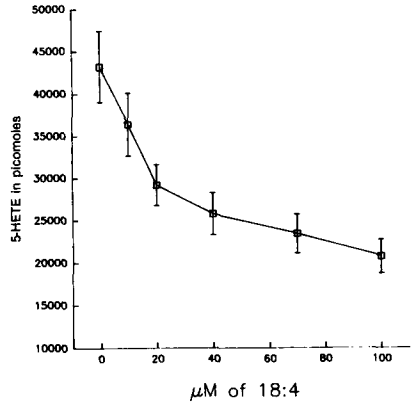

FIG. 6. Effect of 18:4n-3 on 5-hydroxy-eicosatetraenoic acid formation by human leukocytes. One mL leukocytes (10^8 cells/mL) were preincubated in Tyrode *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid containing 2 mM $CaCl_2$ and different concentration of 18:4n-3 (0, 10, 20, 40, 70, 100 μM) for 30 min at 37°C and afterward stimulated with A23187 (1 μM) and 20:4n-6 (10 μM) for 10 min at 37°C. 5-HETE was quantified as described in Materials and Methods. The results are expressed in picomoles per 10^8 cells and represent means ± SEM of three determinations.

(19,24,25). A minor compound which eluted just after LTB_4 (R_t = 29.96 min) was identified as 5*S*,12*S*-[*E*,*Z*,*E*,*Z*-di-HETE. This compound with UV absorptions at λ_{max} 259, 268 and 279 nm has been reported previously (19,24-26).

The effects of the various fatty acids on the formation of metabolites by leukocytes are presented in Figure 5.

In the control, the formation of 5-HETE was maximal but decreased significantly ($P < 0.05$) by about 22% with 20 μM 18:4n-3. More pronounced inhibition was observed with 20 μM 20:5n-3 or 20 μM 20:3n-6, which amounted to 35 and 48%, respectively. Leukotriene formation was affected in a similar way.

Inhibition of LTB_4 was maximal (80%) when leukocytes were incubated with 20 μM 20:3n-6 ($P < 0.001$). LTB_4 formation fell by 50% ($P < 0.001$) in the presence of 20 μM 20:5n-3 or 20 μM 18:4n-3. Similar patterns of inhibition were observed with both of the LTB_4 isomers A and B, especially in the presence of 20:3n-6.

Furthermore, the production of 5*S*,12*S*-di-HETE, formed by synergy between platelets and leukocytes was also decreased but was only significant ($P < 0.001$) with 20 μM 20:3n-6 (75% inhibition).

Figure 6 shows the effect of different concentrations of 18:4n-3 on the formation of 5-HETE by leukocytes stimulated with 1 μM A23187 and 10 μM 20:4n-6. The data show that 18:4n-3 caused a dose-dependent inhibition of 5-HETE formation, and at the highest concentration of fatty acid used (100 μM) it resulted in an inhibition by about 45%.

DISCUSSION

Our results show that PUFAs modulate the level of leukotriene formed *in vitro* by activated leukocytes. Inhibition of leukotriene synthesis by 20:5n-3 and 22:6n-3 had previously been demonstrated (16,27-29), but not inhibition by 18:4n-3. Stearidonic acid is usually only a minor fatty acid in human plasma, and it is not found in many foods. However, after a 16 g/d supplementation with blackcurrant seed oil, which contains about 2 to 3%

18:4n-3, the level of 18:4n-3 can be increased to 0.3% in plasma and also in the blood cells (results not shown). In the present study stearidonic acid exerted an inhibitory effect on arachidonic acid metabolism similar to that observed with 20:5n-3. The production of 5*S*,12*S*-diHETE and 5-HETE were depressed by about 25% by 20 μM 18:4n-3 indicating inhibition of 5-lipoxygenase activity. The synthesis of the two nonenzymatic leukotrienes (isomers A and B) was also decreased to a similar extent. The inhibition of leukotriene B_4 formation was approximately twice that observed for 5-HETE production under the same conditions (Fig. 5). This is consistent with the suggestion that 18:4n-3 may inhibit both 5-lipoxygenase and epoxide hydrolase. Such a mechanism has already been reported for 20:5n-3 (30,31). The authors suggested that a metabolite of 20:5n-3 such as LTA_5 could down-regulate the conversion of LTA_4 to LTB_4 by LTA_4 hydrolase *via* a competitive process. A simplified diagram of the 5-lipoxygenase pathway is given in Figure 1. Moreover, 10 μM 18:4n-3 decreased formation of leukotriene by 35% but not that of 5-HETE (about 5%). Even with 10 μM 18:4n-3, oxygenated products are synthesized by leukocytes. Nevertheless, the metabolism of stearidonic acid appeared to be slower than that of arachidonic acid. Only 3% of the total radioactivity added as [1-^{14}C]stearidonic acid was recovered as hydroxylated compounds.

The inhibition of 5-lipoxygenase by 18:4n-3 was dose-dependent as shown in Figure 6. A similar inhibitory dose response was recently reported for 18:2n-6 and 20:3n-6 which both inhibited LTB_4 formation (32).

Among the fatty acids tested, the most potent inhibitor is 20:3n-6 which almost completely blocks the production of all 5-lipoxygenase products. The formation of LTB_4 and 5-HETE was reduced by 80 and 40%, respectively. This inhibitory effect has also been shown *in vivo* in different models of inflammation (33). Rats fed diets enriched with 18:3n-6, a precursor of 20:3n-6, showed as expected an increase in 20:3n-6 in their liver as well as a marked decrease in prostaglandin E_2 and LTB_4 synthesis (33). These two mediators have been shown to be formed during acute inflammation produced by monosodium urate crystals or subcutaneous air pouch models. Two mechanisms have been proposed to explain this inhibitory effect. When 20:3n-6 is metabolized to prostaglandin E_1, it has been shown to inhibit leukotrienes synthesized by activated human neutrophils (25,34) through an increase in cytosolic cAMP levels in human polymorphonuclear neutrophils (35). Secondly, 20:3n-6 could also be converted into 15-OH-20:3 by leukocyte 15-lipoxygenase and inhibit 5-lipoxygenase as has previously been described (32–36).

In conclusion it appears that stearidonic acid is a good 5-lipoxygenase inhibitor *in vitro* and its effect is comparable to that of timnodonic acid (20:5n-3) at the same concentration. The presence of both stearidonic (2–3%) and γ-linolenic acid (15–19%), a precursor of 20:3n-6 in blackcurrant seed oil (2–3%), suggests that this oil could become useful in the treatment of inflammatory diseases.

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Conversion of 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic Acid to the Corresponding Hydroxy Fatty Acid by KOH: A Kinetic Study

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Transformation of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HPOD) to 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HOD) under alkaline conditions (0.05 to 5 M KOH) occurred first-order with respect to 13*S*-HPOD concentration. Overall yield was about 80%. The energy of activation at higher concentrations (3.75 to 5 M KOH) was determined to be in the range of 15.3 to 15.6 kcal. Compared to the 13*S*-HPOD conversion, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HPOT) was converted at a faster rate to the corresponding hydroxy fatty acid (13*S*-HOT), with the reaction also being first-order. Chiral phase high-performance liquid chromatography demonstrated that in the transformation the stereochemistry of both the 13*S*-HPOD and 13*S*-HPOT reactants was preserved. Manometric analyses of the KOH/13*S*-HPOD reaction showed an uptake of gas, which amounted to 11% of the mols of reactant 13*S*-HPOD on the assumption that the gas was O_2. As there is a theoretical loss of 1 oxygen atom in the reaction, the fate of this oxygen (possibly *via* active oxygen species) may involve reaction with 13*S*-HPOD/13*S*HOD to form the 20% by-products.
***Lipids* 28,** 325–330 (1993).

The base-catalyzed conversion of organic hydroperoxides to alcohols has been known for over thirty years (1). Frankel *et al.* (2) first reported that saponification of methyl linoleate hydroperoxides resulted in formation of the corresponding hydroxy fatty acids. Subsequently, O'Brien (3) found that the conversion of linoleic acid hydroperoxide into the hydroxy fatty acid occurred in the presence of a number of "nucleophiles" other than hydroxide ion, such as dimercaptoethanol, cysteine, ascorbate, thiourea and sulfite. However, some of these reagents may actually serve as reductants. Although the thiolate anion of cysteine appears to be required to convert linoleic acid hydroperoxide to the hydroxy fatty acid, the fact that O_2 is also reduced to superoxide anion under these conditions may indicate the possibility of a hydroperoxide reduction by thiolate anion (4). Although the transformation by alkali has been known for many years, the specific details concerning overall yield, retention (or loss) of stereoconfiguration, mechanism of reaction and reaction kinetics remain little understood. Such information could be important when hydroxy fatty acid methyl esters are found in biological material after use of base in either saponification or transmethylation reactions of lipids (*e.g.*, see Ref. 5). The present communication reports an investigation of the reaction of two pure hydroperoxides, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HPOD) and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HPOT), with KOH at various concentrations.

MATERIALS AND METHODS

Materials. 13*S*-HPOD was prepared from linoleic acid (Nu-Chek-Prep, Inc., Elysian, MN) by oxidation with soybean lipoxygenase [EC 1.13.11.12] (Sigma Chemical Co., St. Louis, MO) using the method of Gardner (6). The 13*S*-HPOD product was isolated by column chromatography with SilicAr CC4 (Mallinkrodt, Inc., St. Louis, MO), and stepwise elution was performed with 5, 7.5 and 10% acetone in hexane (7). Fractions were monitored by absorption of the hydroperoxy diene at 234 nm. The initial 150 mL of absorbing eluent was collected. The solvent was removed on a rotary evaporator, and the reaction product redissolved in methanol. Concentration was determined on a DU-8B spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). Maximum absorbance of the isolated hydroperoxide was at 233 nm, with an extinction coefficient of 26,800 $M^{-1}cm^{-1}$ (8). Purity was checked by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). A hydroxy standard, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HOD), was prepared by reducing 13*S*-HPOD in an excess of sodium borohydride in methanol for 0.5 h at 0°C.

13*S*-HPOT and 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HOT) were prepared in the same manner as was done for 13*S*-HPOD and 13*S*-HOD. Racemic mixtures of methyl hydroxyoctadecadienoates, (*rac*-HOD), and methyl hydroxyoctadecatrienoates, (*rac*-HOT), were produced by sodium borohydride reduction of autooxidized linoleic and linolenic acids as previously described (8).

Kinetics. Stock solutions of fatty acid hydroperoxides in methanol were placed in diethyl ether and taken to near dryness with N_2 to remove methanol. KOH solution was added commencing the alkaline reaction. The system was vortexed 30 s and sonicated 60 s and then incubated in a temperature regulated bath. At intervals, 1-mL aliquots were removed to a separatory flask containing 3.5 mL H_2O, a measured quantity of internal standard, 9,11-octadecadienoic acid (Nu-Chek-Prep) and sufficient citric acid to result in a pH of 4–4.2. Extraction of the product and reactant was performed using 3 vol (based on acidified aqueous solution) of $CHCl_3$/MeOH (2:1, vol/vol). The $CHCl_3$ layer, containing the fatty acids, was evaporated under a stream of N_2 and the material redissolved in hexane/isopropanol (100:2, vol/vol) for HPLC analysis.

HPLC was used to determine the ratio of hydroxide to hydroperoxide fatty acid with a 4.6 × 250 mm Microsorb 5 μm silica column (Rainin Instrument Co., Inc., Woburn, MA). Isocratic runs were made with a Spectra-Physics

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Abbreviations: E_a, energy of activation; EDTA, ethylenediaminetetraacetic acid; 13*S*-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13*S*-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 13*S*-HOD, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 13*S*-HOT, 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; HPLC, high-performance liquid chromatography; *rac*-HOD, racemic mixture of 9(*R,S*)-hydroxy-10(*E*),12(*E,Z*)- and 13(*R,S*)-hydroxy-9(*E,Z*),11-(*E*)-octadecadienoic acids; *rac*-HOT, racemic mixture of 9(*R,S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-, 12(*R,S*)-hydroxy-9(*Z*),13(*E*),15(*Z*),13(*R,S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-, and 16(*R,S*)-hydroxy-9(*Z*),12(*Z*),14(*E*)-octadecatrienoic acids; TLC, thin-layer chromatography.

SP8800 pump (Spectra-Physics, San Jose, CA) using hexane/isopropanol/acetic acid (100:1.5:0.1, by vol) as solvent system. Absorption was monitored with a Spectra-Physics SP8490 detector at 235 nm. Quantities were determined using 9,11-octadecadienoic acid as an internal standard.

Chirality determination. Chirality of hydroxy fatty acids (as their methyl esters) was determined with a Bakerbond Chiral Phase DNBPG (ionic, 5 μm) 4.6 × 250 mm column (J.T. Baker, Phillipsburg, NJ) essentially as described previously (9). Analyses were performed isocratically using a Waters Model 6000A pump (Waters Associates, Inc., Milford, MA) with a solvent system consisting of hexane/isopropanol (99.5:0.5, vol/vol). Absorption was monitored at 235 nm using a Perkin-Elmer LC-75 spectrophotometric detector (Perkin-Elmer, Norwalk, CT). Fatty acids were esterified with diazomethane prior to analysis. Reactant hydroperoxides were examined after first reducing them with $NaBH_4$ (10) followed by esterification with diazomethane.

Respirometer study. A Gilson differential respirometer (Gilson Medical Electronics, Inc., Middleton, WI) was used to examine for gas evolution from mixtures of 13*S*-HPOD in KOH solution using general prescribed techniques (11). Two reaction flasks were used, one as a blank, the other for the reaction. Initially, 13*S*-HPOD was placed in the reaction vessel and the solvents removed. KOH solution was then placed in the vessel sidearm and the vessel fitted to the respirometer and submerged in the bath at 30°C. After temperature equilibration, the manometer was closed and the vessel was tipped, spilling the KOH solution into the vessel. The change in volume of gas was measured *vs.* time.

RESULTS AND DISCUSSION

Product analyses. Conversion of fatty acid hydroperoxides to hydroxide counterparts in alkaline media was noted previously (for a review, see Ref. 12), but neither the kinetics nor the mechanism has been examined. The investigation focused on the kinetics of the reaction and sought to identify its mechanism. Figure 1 shows TLC evidence of the hydroxy fatty acid produced from the reaction of 13*S*-HPOD with KOH. TLC further demonstrated that elevated reaction temperatures and the use of ethylenediaminetetraacetic acid (EDTA) to prevent interference from metal ions did not significantly alter the product composition. Using an internal standard, it was determined that there was an 80% conversion of 13*S*-HPOD into 13*S*-HOD, the remainder presumably consisting of other products.

Previous work has not shown if there is preservation of the stereochemistry in the transformation. Treatment of 13*S*-HPOD with 5 M KOH for 1.1 h at 35°C followed by chiral-phase HPLC of the methyl esters showed that the hydroxy fatty acid product was 96% 13*S*-HOD, with the remainder being 13(*S*)-hydroxy-9(*E*),11(*E*)-octadecadienoic acid (13*S*-*E*,*E*-HOD). This analysis is to be compared with $NaBH_4$-reduced 13*S*-HPOD starting material that was 96% 13*S*-HOD. Figure 2 shows a co-injection of KOH product with a racemic mixture of hydroxy fatty acids (*rac*-HOD) obtained by $NaBH_4$-reduction of autoxidized linoleic acid. The KOH product eluted with the peak obtained from autoxidation and earlier identified as 13*S*-HOD (8,9). Similarly, treatment of 13*S*-HPOT with KOH under the same conditions resulted in the formation of

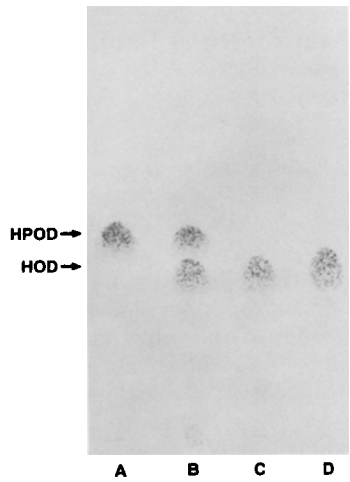


FIG. 1. Thin-layer chromatography separation of 13*S*-HPOD and 13*S*-HOD using a pre-coated Silica Gel 60-F-254 plate. Solvent system hexane/diethyl ether/HOAc (50:50:1, by vol): A, 13*S*-HPOD reactant; B, result of 2.3×10^{-3} M 13*S*-HPOD in 1 M KOH, 5 mM EDTA for 23 h at room temperature (ratio of HOD to HPOD, 83:17, by HPLC); C, result of 2.3×10^{-3} M 13*S*-HPOD in 5 M KOH, 5 mM EDTA for 5 h at 35°C (ratio of HOD to HPOD, 99:1, by HPLC); and D, 13*S*-HOD derived from 13*S*-HPOD by $NaBH_4$ reduction. 13*S*-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13*S*-HOD, 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

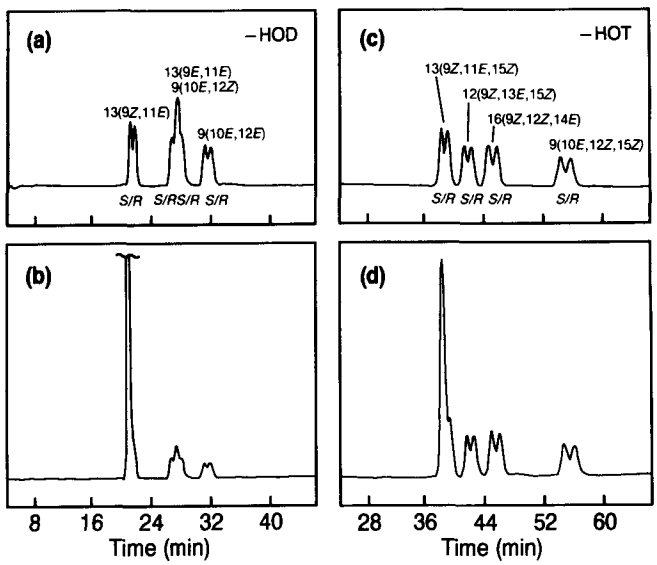


FIG. 2. HPLC separation of *rac*-HOD and *rac*-HOT and co-injection of racemic mixtures with 13*S*-HOD and 13*S*-HOT derived from KOH-treatment of 13*S*-HPOD and 13*S*-HPOT. Separations were performed on a chiral column (250 × 4.6 mm) at ambient temperature with a mobile phase of hexane/isopropanol (99.5:0.5, vol/vol) and a flow rate of 1 mL/min for (a) and (b) and 0.624 mL/min for (c) and (d); detection was by measuring absorbance at 234 nm. (a) Eight isomers of 9- and 13-hydroperoxides of *rac*-HOD with *R* and *S* stereoconfiguration [13*R*,*S*-HOD(9*E*,11*E*) and 9*R*,*S*-HOD (10*E*,12*Z*) peaks overlap]; (b) product of KOH treated 13*S*-HPOD co-injected with *rac*-HOD; (c) eight isomers of 9-, 12-, 13- and 16-hydroperoxides of *rac*-HOT with *R* and *S* stereoconfiguration (Ref. 9); and (d) product of KOH treated 13*S*-HPOT coinjected with *rac*-HOT. Abbreviations as in Figure 1. *rac*-HOD, racemic mixture of 9(*R*,*S*)-hydroxy-10(*E*),12(*E*,*Z*)- and 13(*R*,*S*)-hydroxy-9(*E*,*Z*),11(*E*)-octadecadienoic acids; *rac*-HOT, racemic mixture of 9(*R*,*S*)-hydroxy-10(*E*),12(*Z*),15(*Z*)-, 12(*R*,*S*)-hydroxy-9(*Z*),13(*E*),15(*Z*)-, 13(*R*,*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)- and 16(*R*,*S*)-hydroxy-9(*Z*),12(*Z*),14(*E*)-octadecatrienoic acids.

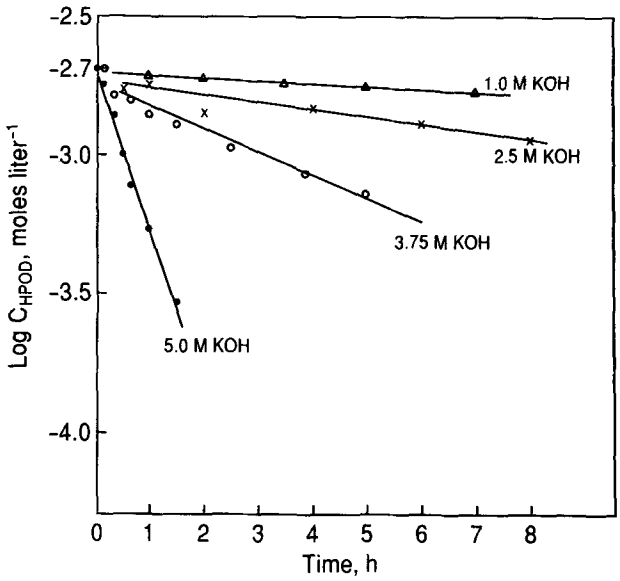


FIG. 3. First order plots of 13*S*-HPOD conversion (2.0×10^{-3} M starting concentration) at various concentrations of KOH at 35°C. Abbreviations as in Figure 1.

86% 13*S*-HOT as compared with 100% 13*S*-HOT obtained by $NaBH_4$-reduction of 13*S*-HPOT. The remaining 14% was an unidentified reaction product that did not correspond by chiral analysis to any of the racemic mixture of hydroxyoctadecatrienoic acid (*rac*-HOT) isomers. Figure 2 also reveals that a co-injection of the KOH-treated 13*S*-HPOT with *rac*-HOT showed the main product had the same retention time as 13*S*-HOT. These results lead to the important conclusion, regarding the mechanism, that the reaction does not proceed by nucleophilic substitution, which would either invert or randomize the stereoconfiguration.

Reaction kinetics. Examination of the reaction began by determining the reaction order for 13*S*-HPOD conversion at high KOH concentrations. The reaction was followed by plotting the log of the hydroperoxy concentration *vs.* time. Most of the kinetics were determined for 13*S*-HPOD and the data are presented in Figure 3. As seen in Table 1, four KOH concentrations were used at a fixed molarity of 2.3×10^{-3} 13*S*-HPOD. Three different temperatures were used, and the rate constants were calculated for a first-order reaction.

Plotting Arrhenius energies of activation in Figure 4 for the reactions provided evidence of the reaction difference between the 1.0 and 2.5 M KOH solutions *vs.* the higher KOH concentrations. Between 1.0 and 3.75 M KOH there was a decreasing energy of activation with increasing KOH concentration. In contrast, at either 3.75 or 5.0 M KOH, where much faster reaction rates occurred, the energy of activation (E_a) values were essentially the same.

The question whether KOH was participating in the reaction or acting as a catalyst needed to be determined. If KOH was involved in the reaction, the reaction would not be first-order. To examine for KOH involvement required that the alkali concentration be just above that of the fatty acid, because it must saponify the lipid and provide a basic medium, yet be of a low enough concentration to affect a change in KOH concentration with reaction time. The solution used to determine the KOH reaction order was 0.05 M KOH and 0.023 M 13*S*-HPOD. Assuming complete saponification, the remaining KOH concentration was 0.031 M, providing a pH of 12.5. Resultant conversion curves at 35 and 45°C are presented in Figure 5. For the purpose of comparison, curves representing the percent conversion of hydroperoxide material to its hydroxy form were plotted *vs.* time. Each reaction was

TABLE 1

Reaction Rates for 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic Acid (13*S*-HPOD) Reduction to 13(*S*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic Acid in KOH Solutions at Various Incubation Temperatures[a]

KOH concentration	Temp. (°C)	Reaction rate K (s^{-1}, $\times 10^{-5}$)
1 M	35	0.72
	45	3.0
	55	11
2.5 M	35	1.1
	45	4.2
	55	13
3.75 M	35	4.9
	55	23
5 M	24	13
	35	34
	45	60
0.05 M	35	5.6
	45	10

[a]The concentration of 13*S*-HPOD was 2.3×10^{-3} M, except for the 0.05 M KOH solution where it was 2.3×10^{-2} M.

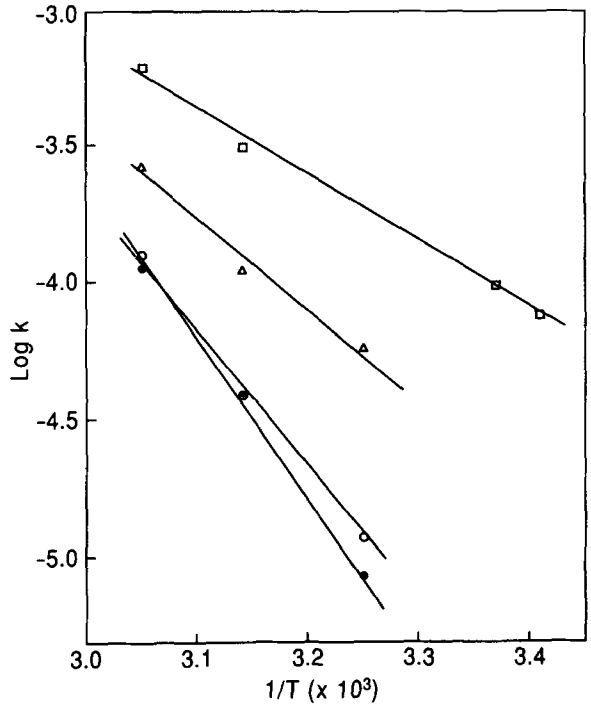


FIG. 4. Arrhenius plot of log reaction rates *vs.* reciprocal of temperature for 2.3×10^{-3} M 13*S*-HPOD in 1 M KOH, E_a (cal. $mole^{-1}$) = 26,000 (□); 2.5 M KOH, E_a = 23,500 (△); 3.75 M KOH, E_a = 15,300 (○); and 5.0 M KOH, E_a = 15,600 (●). E_a, energy of activation. Other abbreviations as in Figure 1.

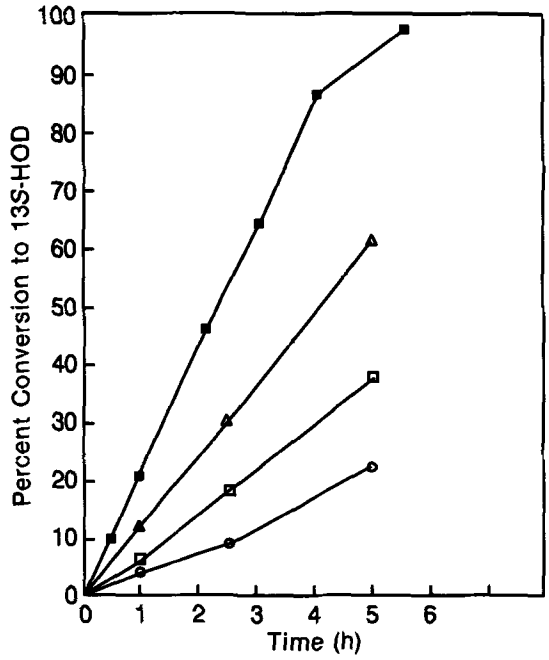


FIG. 5. Time course of 13S-HOD formation in 0.05 M KOH with varying starting concentrations of 13S-HPOD and temperatures. 13S-HPOD concentrations and incubation temperatures are 3.9×10^{-3} M, 35°C plus 1.8×10^{-2} M palmitic acid (total fatty acid, 2.2×10^{-2} M) (○); 3.9×10^{-3} M, 35°C (□); 2.3×10^{-2} M, 35°C (△); and 2.3×10^{-2} M, 45°C (■). Abbreviations as in Figure 1.

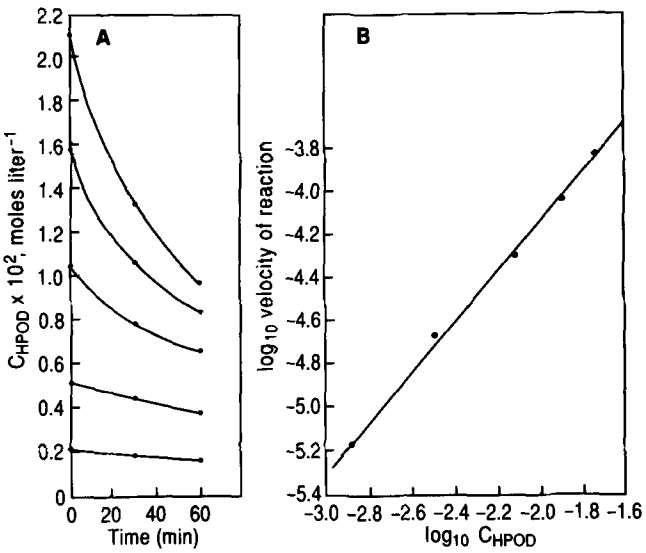


FIG. 6. A. Effect of 13S-HPOD concentration on the rate of conversion into 13S-HOD. Initial concentrations are 2.1, 1.58, 1.05, 0.52 and 0.21 M ($\times 10^{-2}$) 13S-HPOD. Reactions were by incubation for 1 h at 35°C in 2.0 M KOH. Rates of conversion determined tangentially at 30 min. B. Log-log plot of conversion rate *vs.* concentration. Abbreviations as in Figure 1.

first-order with respect to the hydroperoxide. Comparison of these rates to higher alkaline concentration runs are given in Table 1. The reaction rate at lower KOH concentration, 0.05 M, was proportional to the rates at the higher concentrations. A projected reaction rate of 0.64×10^{-5} s^{-1} was derived by extrapolating the reaction rates from Table 1 for 2.5 and 1.0 M KOH at 35°C and 2.3×10^{-3} M 13S-HPOD solutions to 0.05 M KOH. This value was then multiplied by a factor 10 to correct for the increase in hydroperoxide concentration to the 2.3×10^{-2} M 13S-HPOD at 0.05 M KOH. The value of 4.5×10^{-5} s^{-1} is close to the 5.6×10^{-5} s^{-1} value experimentally obtained under these conditions (Table 1). With the extrapolation yielding a slope of 1, the rate of hydroperoxide activity is directly proportional to OH^- concentration at KOH molarity up through 2.5. The conclusion is that KOH is not part of the reaction, but performs as a basic catalyst.

Rate dependence on 13S-HPOD concentration. The effect of hydroperoxide fatty acid concentration on the reaction rate was examined using varying concentrations of 13S-HPOD at constant KOH (2.0 M) at 35°C (Fig. 6). The lipid concentrations were 10^{-3} to 10^{-2} less than the KOH concentration. Reactions were examined at 30 and 60 min. The velocity of a reaction at 30 min was obtained from the slope of the tangent to the curve at 30 min. The reaction order was obtained by plotting the $\log_{10}$ velocity *vs.* $\log_{10}c_{HPOD}$. A linear regression analysis provided a line having a slope of 1.1, which is confirmatory of a first-order reaction.

The presence of nonhydroperoxy lipid material, such as palmitic acid, slowed the conversion of hydroperoxy compounds into their corresponding hydroxides (Fig. 5). Presumably, the addition of palmitic acid resulted in the formation of mixed micelles, thus masking the 13S-HPOD within a fatty acid micelle.

Temperature dependence. The initial rates approximately doubled for every ten degree increase in temperature. This observation was in keeping with reaction rate increase behavior for most chemical reactions near room temperature. There was no evidence that changing the incubation temperature altered the mechanism or the kinetic patterns at any of the KOH or hydroperoxide fatty acid concentrations tested.

Rate comparison of 13S-HPOD and 13S-HPOT. The rates of conversion of 13S-HPOD and 13S-HPOT to the corresponding hydroxy fatty acids were compared under two differing sets of conditions. The two trials differed in KOH concentration and incubation temperature (Fig. 7). Under both sets of conditions, the rate of product formation for the triene was greater than for the diene. 13S-HOT formation rates were first-order, although it was observed that in 5 M KOH the reaction rate decreased more rapidly after about 50% reaction completion than the rate for 13S-HPOD (data not shown). On the other hand, aliquots taken immediately after mixing of 13S-HPOT and KOH resulted in an unexplained rapid depletion of 13S-HPOT (Fig. 7), which was accounted for as conversion into 13S-HOT (data not shown). Other competing reactions may be the cause of the difference in 13S-HPOT kinetics, indicating the need for future work.

The hydroperoxide oxygen. The fate of the hydroperoxide oxygen is critical to determining the mechanism of the reaction. Formation of hydrogen peroxide as a possible reaction by-product was examined. The lack of peroxide formation was demonstrated using the AOCS method of peroxide value determination (13). A 13S-HPOD solution, 1.1×10^{-1} milliequivalents (meq)/mL 2 M KOH, was incubated at room temperature for 3 h. The resulting solu-

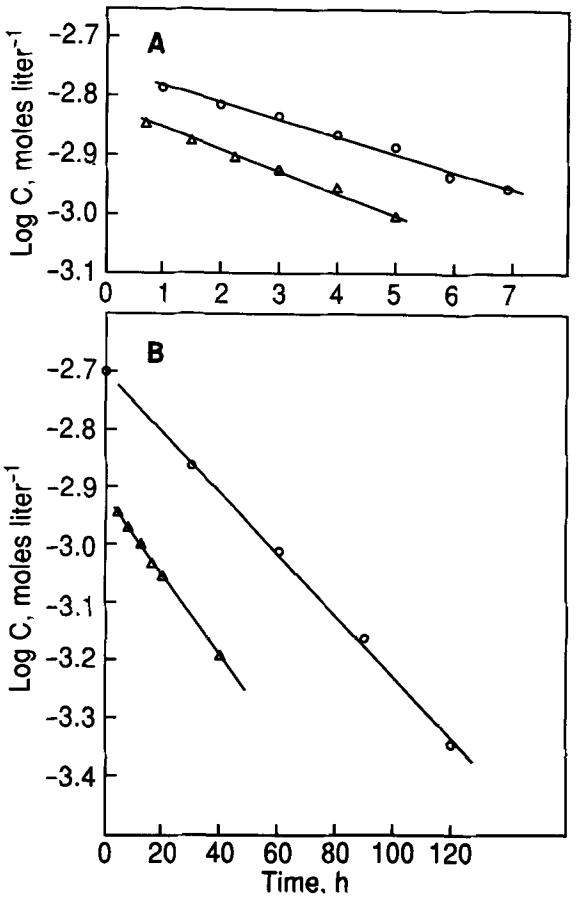
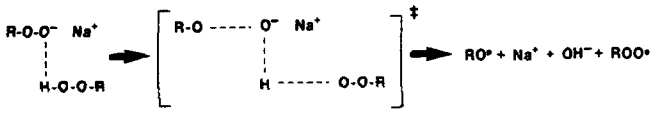


FIG. 7. Plot of log hydroperoxide concentration *vs.* incubation time for comparison of the conversion of 13*S*-HPOT (△) *vs.* 13*S*-HPOD (○). 13*S*-HPOT and 13*S*-HPOD starting concentrations were 2.0×10^{-3}. Incubation conditions: (A) 2.5 M KOH at 35°C; and (B) 5 M KOH at 25°C. Abbreviations as in Figure 1.

tion, following acidification, yielded a maximum peroxide value of 4×10^{-6} meq/mL. This is a factor of 10^{-4} times lower than the 5.5×10^{-2} meq/mL expected, further casting doubt on either a possible conversion of 13*S*-HPOD to H_2O_2 or its persistence in the alkaline medium. However, we cannot discount the possibility that H_2O_2 is a fleeting intermediate in the reaction.

Another possibility for the loss of oxygen in the hydroperoxide conversion to hydroxide is that the hydroperoxide oxygens could be released as molecular oxygen. A differential respirometer was used to examine for oxygen evolution during 13*S*-HPOD conversion to 13*S*-HOD. 13*S*-HPOD (33 μmol) was reacted with 0.5 mL of 5 M KOH in a respirometer reaction vessel, giving an uptake of 82 μL of gases during a 2-h period of incubation. In terms of oxygen (O_2), 3.6 μmol of gas was consumed. On a basis of reactivity of 1 O_2 per 13*S*-HPOD molecule, 11% of the hydroperoxide reacted with oxygen. This O_2 uptake may explain the 20% non13*S*-HOD by-products resulting from the reaction. Indeed, the by-products found arise from a dioxygenated intermediate (Gardner, H.W., and Hamberg, M., personal communication). However, these data do not explain the fate of one-half mol of O_2 expected to be produced from loss of one hydroperoxide oxygen in the reaction. Denny and Rosen (14) also observed oxygen consumption in treating 1,1,3,3,-tetramethylbutyl hydroperoxide in 10% NaOH.

The "Russell" mechanism for the reaction of secondary hydroperoxides by combination of peroxyl radicals was proposed in 1957 (15). Later, Howard and Ingold (16) and Nakano *et al.* (17,18) extended and further clarified this mechanism in the oxidation studies of *sec*-butyl hydroperoxide and linoleic acid hydroperoxide using ceric ion (Ce^{+4}). Of significance in this mechanism was the resultant production of an alcohol, a ketone and singlet oxygen. Denny and Rosen (14) also suggested a bimolecular reaction in the reduction of cumyl hydroperoxide:



However, it appears unlikely that the 13*S*-HPOD to 13*S*-HOD conversion reaction proceeds by a bimolecular reaction. In general, the order of a reaction is related to the sum of the exponentials of the reactant(s) in the rate equation. A bimolecular reaction would be expected to be second-order.

Accountability of the oxygen removed in the hydroperoxide conversion remains the puzzle. If one ignores the observed O_2 uptake data, the 20% by-products can only account for 20% of oxygen assuming each molecule reacts with one atomic oxygen plus retaining its own initial oxygen.

Simply put, we believe the conversion of 13*S*-HPOD to 13*S*-HOD in KOH does not proceed by a bimolecular reaction involving two hydroperoxide molecules nor does it proceed by a simple first-order reaction, although the kinetic results show the rate is proportional to the hydroperoxide concentration. The present inexplicability of the oxygen destination indicates a much more complex reaction mechanism, one which requires further investigation.

13*S*-HOD has been reported as a vessel wall chemorepellant factor (19) and as a factor in the self-defense mechanism in rice plants against rice blast disease (20). Because of its apparent active role in modulating cellular functions, two recent reports have dealt with the synthesis of 13*S*-HOD (coriolic acid) (21,22). Neither invoked use of the hydroperoxy precursor. It has been demonstrated here that an alkaline conversion of 13*S*-HPOD or 13*S*-HPOT can yield chirally pure 13*S*-HOD and 13*S*-HOT, respectively, in reasonably high yield. Optimum conditions for complete conversion have not yet been established.

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Identification of Core Aldehydes Among *in vitro* Peroxidation Products of Cholesteryl Esters

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Synthetic cholesteryl 5-oxovalerate and 9-oxononanoate were used as reference standards for the isolation and identification of cholesteryl ester core aldehydes from *tert*-butyl hydroperoxide/Fe^{++} oxidation of synthetic and natural cholesteryl esters. The core aldehydes were recovered from the peroxidation products by thin-layer chromatography as the free aldehydes or the 2,4-dinitrophenylhydrazones and were identified, respectively, by gas-liquid chromatography (GLC) and by GLC combined with mass spectrometry (GC/MS) or by reverse-phase high-performance liquid chromatography (HPLC) and by HPLC with MS (LC/MS). The core aldehydes produced by peroxidation of cholesteryl linoleate were identified as mainly 9-oxononanoates of cholesterol and oxycholesterols, with smaller amounts of the 8-oxooctenoates, 10-oxodecenoates, 11-oxoundecenoates and 12-oxododecenoates. Peroxidation of cholesteryl arachidonate yielded 5-oxovalerates of cholesterol and the oxycholesterols as the main products with smaller amounts of the 4-oxobutyrates, 6-oxohexenoates, 7-oxoheptenoates, 8-oxooctenoates, 9-oxononenoates, 9-oxononadienoates and 10-oxodecadienotes. The oxycholesterols resulting from the peroxidation of the steroid ring were identified as mainly 7-keto-, 7α-hydroxy- and 7β-hydroxy-cholesterols and 5α,6α- and 5β,6β-epoxy-cholestanols. Cholesteryl palmitate and oleate did not yield core aldehydes in the present peroxidation system. In these esters, the sterol and linoleic acid moieties appeared to be oxygenated at about the same rate, while the arachidonic acid moiety reacted more rapidly than did the sterol moiety.
Lipids 28, 331-336 (1993).

There is extensive evidence that the autoxidation products of cholesterol and cholesteryl esters exert pronounced biological effects. Thus, the products of autoxidation of free cholesterol are well known inhibitors of the rate-limiting enzymes in cholesterol and bile acid biosynthesis (1). In addition, the cholesterol oxides possess carcinogenic (2) and atherogenic (3) properties. Among the major peroxidation products of cholesterol found in human plasma are the isomeric cholesterol 5α,6α- and 5β,6β-epoxides (4). The autoxidation products of cholesteryl esters have been less thoroughly investigated, and there is evidence that the fatty chain may exert a protective effect on the autoxidation of the steroid ring (5,6). Previous work has demonstrated the presence of cholesteryl ester hydroperoxides in fresh human blood plasma (7), but specific molecular species have not been identified. Stored human blood plasma has been shown to contain 9- and 13-hydroxylation products of cholesteryl linoleate and arachidonate (8), which presumably originated from a reduction of the corresponding hydroperoxides. In the present study, we have demonstrated the formation of cholesteryl ester core aldehydes during *in vitro* peroxidation of synthetic and natural cholesteryl esters with *tert*-butyl hydroperoxide in the presence of $FeSO_4$, which is believed to mimic lipid peroxidation in natural systems (9). This is the first report of the occurrence of lipid soluble core aldehydes among the peroxidation products of cholesteryl esters.

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Abbreviations: CI, chemical ionization; DNPH, dinitrophenylhydrazone; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MOX, methoxime; NCI, negative chemical ionization; *t*-BOOH, *tert*-butyl hydroperoxide; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet.

MATERIALS AND METHODS

Chemicals and reagents. Cholesteryl palmitate, oleate, linoleate and arachidonate, and *tert*-butyl hydroperoxide (*t*-BOOH) and cholesteryl ester hydrolase *(Pseudomonas)* were obtained from Sigma Chemical Co. (St. Louis, MO). Methoxylamine HCl (MOX reagent), trimethylchlorosilane and *bis*(trimethylsilyl)trifluoroacetamide were from Pierce Chemical Co. (Rockford, IL), while 2,4-dinitrophenylhydrazine was from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile and 2-propanol [high-performance liquid chromatography (HPLC) grade] were from Fisher Scientific Co. (Toronto, Ontario, Canada) or Caledon Laboratories Ltd. (Georgetown, Ontario, Canada), while propionitrile was from Romil Ltd. (Loughborough, England). All other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers. Standard 5-oxovalerate and 9-oxononanoate of cholesterol were previously synthesized in our laboratory (10). The cholesteryl esters of rat plasma were isolated by thin-layer chromatography (TLC) using the methods described below.

Peroxidation with t-BOOH/$FeSO_4$. The peroxidation routine was modeled after previous work of Borowitz and Montgomery (11). One to two mg of cholesteryl ester in 100 μL of chloroform/methanol (2:1, vol/vol) were placed in a 15-mL test tube, and the solvent was evaporated under nitrogen. To the dry sample was then added 1 mL of 70% aqueous *t*-BOOH and 100 μM $FeSO_4$ and the tube was mechanically shaken for 3 h at 37°C in the dark. The reaction was stopped by diluting the mixture with five volumes of chloroform/methanol (2:1, vol/vol) and washing with water to remove excess *t*-BOOH. The lower layer was collected, evaporated to dryness under nitrogen and taken up in chloroform for purification and identification of core aldehydes.

Isolation of core aldehydes. The chloroform extracts of the reaction mixture were resolved by TLC on silica gel H using heptane/isopropyl ether/glacial acetic acid (60:40:4, by vol) as the developing solvent. The location of the core aldehydes was revealed by spraying the plate with Schiff's reagent (10). The other lipids on the TLC plate were located under ultraviolet (UV) light by spraying the plate with 2,7-dichlorofluorescein. The aldehydes were recovered from the silica gel by extraction with chloroform/methanol (2:1, vol/vol). The core aldehydes and the derived oxycholesterols were reduced with sodium

borohydride to convert any 7-keto- to the 7-hydroxycholesterol derivatives (8).

Preparation of 2,4-dinitrophenylhydrazone (DNPH) derivatives. The cholesteryl ester core aldehydes were converted into the hydrazones using a modification of the method described by Esterbauer and Cheeseman (12) for water-soluble aldehydes. The chloroform extracts of the peroxidation products were treated with 0.5 mL of freshly prepared DNPH reagent (2,4-DNPH in 1 N HCl at a concentration of 0.5 mg/mL), mixed vigorously and kept in dark for 2 h at room temperature and then for 1 h at 4°C. The reaction mixture was extracted with chloroform/methanol (2:1, vol/vol), and the recovered hydrazones purified by TLC using a double development with dichloromethane (10 cm) and, after solvent evaporation, with toluene (17 cm). The yellow zones were scraped off and the gel extracted with methanol. The hydrazones could be purified further by reverse-phase HPLC and the UV absorbing fractions collected.

Enzymatic hydrolyses. The cholesteryl and oxycholesteryl esters were hydrolyzed with cholesteryl ester hydrolase using a method modified from Heider and Boyett (13). The esters (max. 1 mg) were dissolved in sodium taurocholate (25 mM final concentration) and incubated with cholesteryl ester hydrolase (0.5 U/mL) in sodium phosphate buffer (pH 7.4) (1 mL final volume) for 60 min at 37°C. The released sterols were extracted with chloroform/methanol (2:1, vol/vol) and backwashed with water to remove the bulk of the detergent. The oxysterols were purified by TLC using heptane/isopropyl ether/acetic acid (60:40:4, by vol) as solvent.

Gas-liquid chromatography (GLC) and gas chromatography/mass spectrometry (GC/MS). Intact cholesteryl esters, core aldehydes and their trimethylsilyl (TMS) and methoxime (MOX) derivatives were resolved on the basis of their molecular weights by GLC using an 8 m × 0.32 mm i.d. capillary column coated with DB-5 as previously described (10). The TMS derivatives of oxidized sterols were separated on 30 m × 0.32 mm i.d. capillary column coated with DB-5 according to the method of Maerker and Unruh (15). The sterols were injected on-column at 40°C, and after 0.5 min the oven temperature was first increased to 150°C at 30°C/min then to 230°C at 20°C/min, then to 260°C at 10°C/min and finally to 300°C at 0.5°C/min. The carrier gas was hydrogen at 6 psi and the flame-ionization detector was set at 325°C. The sterols were identified by means of standards and by GC/MS in the positive ion mode (16). The MOX derivatives were prepared and silylated as described by Horning *et al.* (17). The MOX derivatives of the core aldehydes were purified by TLC as previously described (10).

HPLC and liquid chromatography/mass spectrometry (LC/MS). Reverse-phase HPLC of the 2,4-DNPHs of the cholesteryl ester core aldehydes was performed on a Supelcosil LC-18 column (250 mm × 4.6 mm i.d.) (Supelco, Inc., Mississauga, Ontario, Canada) using acetonitrile/2-propanol (4:1, vol/vol) (1 mL/min) or a linear gradient of 30–90% propionitrile in acetonitrile (1.5 mL/min) as the eluting solvents as previously described (10). Earlier described methods were also used for reverse phase LC/MS of the DNPH derivatives of core aldehydes (14).

Quantitation. The relative proportions of the various oxidation products were estimated by high temperature GLC following preparation of the TMS-MOX derivatives or the TMS derivatives of the sodium borohydride reduction products of the total lipid extracts of the reaction mixtures (8).

RESULTS AND DISCUSSION

Identification of sterol ring oxidation products. Figure 1 shows the TLC resolution of the peroxidation products of free cholesterol and cholesteryl palmitate and oleate. The free sterol yields two bands, one of which corresponds to residual cholesterol (R_f 0.30) the other one to 7-ketocholesterol (R_f 0.10). Both cholesteryl palmitate and cholesteryl oleate give a band for residual esters (R_f 0.90) and a strong band for a polar product (R_f 0.72). Since the saturated fatty acid is inert to peroxidation, the oxidative transformation responsible for the slower migration must have taken place on the steroid ring. Likewise, the cholesteryl oleate appeared to be oxidized only in the ring, although a minor TLC band was seen corresponding to the 9-oxononanoate of 7-ketocholesterol. This conclusion was confirmed by hydrolysis of the *t*-BOOH oxidation products of cholesteryl oleate with cholesteryl ester hydrolase. The released free sterols gave two TLC fractions, one of which was due to unoxidized, the other to oxidized cholesterol as noted for the oxidation products of free cholesterol.

Figure 2 shows the GLC resolution of the oxycholesterols released by cholesteryl ester hydrolase from peroxidized cholesteryl oleate. These separations were carried out on a 30-m capillary column, which resolved close isomers of oxycholesterol. Specifically identified were 7-keto-, 7α- and 7β-hydroxy- and the 5α,6α- and 5β,6β-epoxy derivatives of cholesterol. The presence of 7-ketocholesterol was confirmed by sodium borohydride reduction, which yielded the 7α- and 7β-hydroxy cholesterols in equal

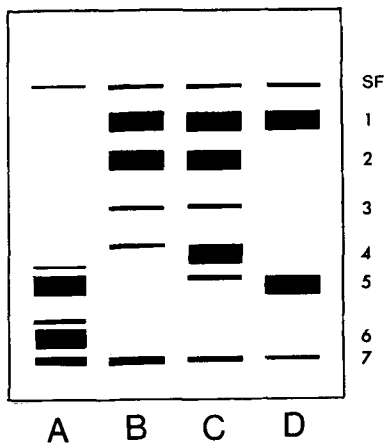

FIG. 1. Thin-layer chromatography resolution of *tert*-butyl hydroperoxide oxidation products of cholesterol, cholesteryl palmitate and cholesteryl oleate (in absence of $FeSO_4$). Lane A, oxidation products of free cholesterol; Lane B, oxidation products of cholesteryl palmitate; Lane C, oxidation products of cholesteryl oleate; Lane D, standard free cholesterol and cholesteryl oleate. Band 1, residual cholesteryl ester; Band 2, 7-ketocholesteryl esters; Band 3, unidentified; Band 4, 7-hydroxycholesteryl esters; Band 5, free cholesterol; Band 6, 7-ketocholesterol; Band 7, origin and 7-hydroxycholesterol. Solvent system: heptane/diisopropyl ether/glacial acetic acid (60:40:4, by vol). Lipid fractions were located by spraying with 2',7'-dichlorofluorescein and Schiff's reagent (10). SF, solvent front.

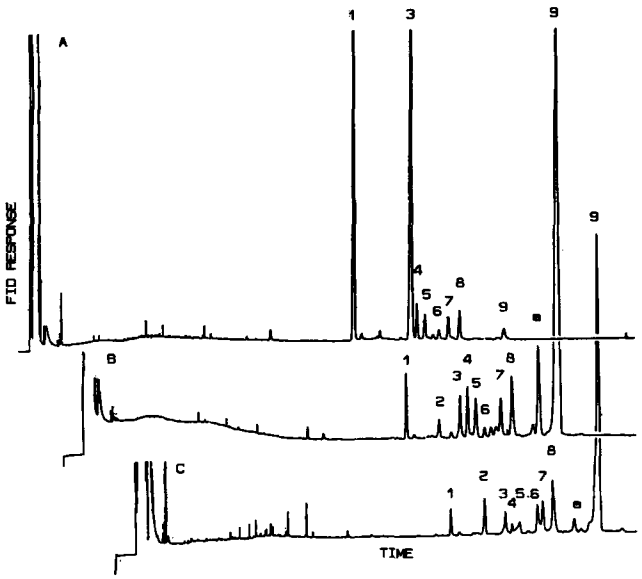

FIG. 2. Gas-liquid chromatography (GLC) resolution of the oxidation products of free cholesterol and cholesteryl oleate. A, oxidation products of free cholesterol after sodium borohydride reduction; B, oxidation products of free cholesterol; C, oxycholesterols released by cholesteryl ester hydrolase from oxidized cholesteryl oleate. Peak identification: 1, 7α-hydroxycholesterol; 2, 3,5-cholestadienol; 3, 7β-hydroxycholesterol; 4, cholesterol 5,6β-epoxide; 5, cholesterol 5,6α-epoxide; 6-8, unidentified oxysterols; 9, 7-ketocholesterol. GLC conditions: 30 m × 0.32 mm capillary coated with DB-5 (10); temperature programmed as given under Materials and Methods. Other GLC conditions, see also Materials and Methods. FID, flame-ionization detector.

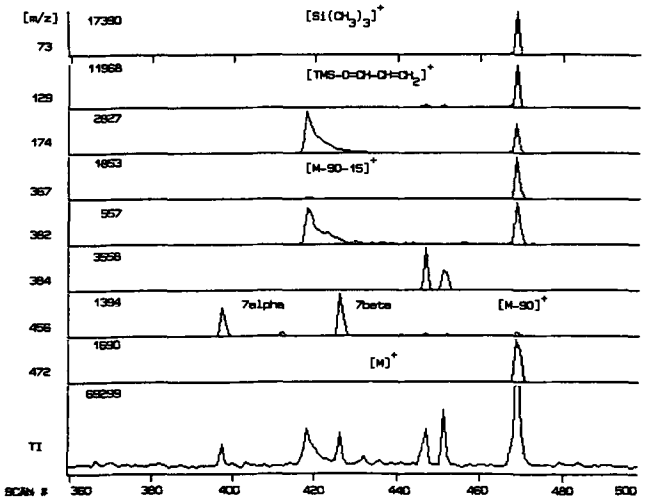

FIG. 3. Total ion current profile and single ion plots for major molecular species of ring oxidized cholesteryl oleate as obtained by gas-chromatography/mass spectrometry (GC/MS) of the oxysterols released by cholesteryl ester hydrolase digestion from oxidized cholesteryl oleate (trimethylsilyl ethers). Peak identification: TI, total positive ion current; *m/z*, fragment ions as identified in the figure. Numbers inside figure refer to the ion counts of the major single ion peak. GC/MS conditions as given under Materials and Methods.

proportions. These diols overlapped with the corresponding minor diol peaks in the original GLC profiles of each ester. Other minor peaks (Peaks 6-8) were attributed to mixed function derivatives but were not further identified. Addition of $FeSO_4$ to the peroxidation medium had no significant qualitative effect on the oxycholesterol composition.

Figure 3 shows the total ion current profile and the single ion plots for the major molecular species of the ring oxidized cholesterol moiety released by the enzyme from the oxidized cholesteryl oleate. The 7α- and 7β-hydroxy cholesterols gave strong $[M - 90]^+$, $[M - 90 - 15]^+$ as well as other minor ions characteristic of the TMS ether. An $[M]^+$ ion at *m/z* 382 was also obtained for cholesta-3,5-dien-7-one, which was assumed to be a minor product of dehydration of 7-ketocholesterol in the gas chromatograph (18). The unknown yielded strong ions corresponding to *m/z* 384, which suggested that they were isomeric dehydration products of oxycholesterol. The mass spectra of the oxysterols agreed with the corresponding spectra recorded in the literature for 7-ketocholesterol (19), 3,5-cholestadien-7-one (20), and the 7α- and 7β-hydroxy cholesterol di-TMS derivatives (21). The GC/MS profiles of the sterol moieties of peroxidized cholesteryl esters were similar to those of the oxysterols recovered from the peroxidation of free cholesterol.

The oxidized cholesteryl palmitate and oleate gave excellent GLC profiles (chromatograms not shown). The oxidized esters were eluted with retention times about one to two methylene units higher than the corresponding unoxidized esters. The overall elution patterns were similar for both esters as would be anticipated from the free oxysterol analyses. Reduction of the 7-ketocholesteryl oleate by sodium borohydride gave the corresponding 7α- and 7β-hydroxy cholesteryl stearates, which upon trimethylsilylation gave the anticipated decreases in the GLC retention times. The peak identity was confirmed by GC/MS, which yielded the correct molecular weights of the esters and fragment ions characteristic of the steroid ring.

TLC separation of the peroxidation products of cholesteryl linoleate gave several bands (chromatogram not shown). In addition to the residual ester (R_f 0.84), there was a band containing oxidized cholesterol in combination with unoxidized linoleic acid (R_f 0.72), a band containing mainly cholesteryl 9-oxononanoate (R_f 0.37) and a more diffuse band made up mainly of 9-oxononanoyl esters of various oxycholesterols (R_f 0.15-0.29). TLC separation of the peroxidized arachidonate failed to yield any of the original ester, but some oxycholesteryl arachidonate may have been present (see below). The identified peroxidation products of cholesteryl arachidonate were made up of mainly 5-oxovaleroyl colesterol (R_f 0.30) and 5-oxovaleroyl esters of the various oxycholesterols (R_f 0.10-0.20). The major steryl ester core aldehydes yielded GLC peaks which corresponded to standard 9-oxononanoyl and 5-oxovaleroyl esters of cholesterol when run as the MOX-TMS ethers or TMS ethers. The GLC peaks of the core aldehyde esters of the oxycholesterols gave much more complex profiles, although the 7-ketocholesterol and cholesteryl epoxide derivatives could be discerned. In all instances, characteristic peak shifts were seen in the GLC retention times when the MOX-TMS and TMS derivatives were analyzed (chromatograms not shown). However, many minor peaks remained unidentified in the mass profiles of the chromatograms, while the mass spectra of these derivatives were compromised by overlapping esters other than lipid ester core aldehydes.

This difficulty was largely overcome by the preparation of the DNPH derivatives of the steryl ester core aldehydes, which could be specifically detected as yellow bands on TLC, UV absorbing peaks on HPLC and negative ions on LC/MS.

Figure 4 shows the TLC separation of the yellow-colored DNPH derivatives of the core aldehydes derived by peroxidation of cholesteryl linoleate (Lane D) and cholesteryl arachidonate (Lane E). The peroxidation products of both esters gave four well-resolved bands (R_f values 0.20–0.40) migrating near the middle of the TLC plate along with less well-resolved yellow regions near the origin. The center bands corresponded in R_f values to the DNPH derivatives of standard 9-oxononanoyl and 5-oxovaleroyl esters of cholesterol. The region near the origin (R_f values 0.10 and 0.20) contained the DNPH derivatives of oxycholesteryl ester core aldehydes. The major TLC fractions were identified by LC/MS with NCI. Figure 5A gives the HPLC profiles of the core aldehydes recovered from the linoleate oxidation. Band 1 (Fig. 4, Lane D) contained mainly cholesteryl 12-oxododecenoate, 11-oxoundecenoate and 10-oxodecenoate. Band 2 (Fig. 4, Lane D) contained mainly cholesteryl 8-oxononanoate, while Band 3 (Fig. 4, Lane D) contained mainly cholesteryl 8-oxooctanoate, 10-oxodecenoate and 10-oxodecanoate. Band 4 (Fig. 4, Lane D) contained an unknown oxycholesteryl 9-oxononanoate, which gave the same molecular ion as 7-ketocholesteryl 9-oxononanoate. The bands near the origin (Fig. 4, Lane D) contained mainly 7-ketocholesteryl 9-oxononanoate and the 9-oxononanoate of 5,6-epoxycholestanol. Figure 5B gives the HPLC profiles of the core aldehydes derived from cholesteryl arachidonate and recovered from the four TLC bands (Fig. 4, Lane E) of the DNPH derivatives. The arachidonate yielded the corresponding

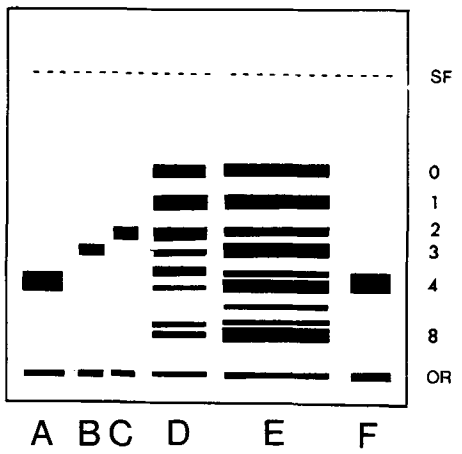

FIG. 4. Thin-layer chromatography (TLC) resolution of dinitrophenylhydrazone (DNPH) derivatives of *tert*-butyl hydroperoxide oxidation products of cholesteryl linoleate and cholesteryl arachidonate. Lane A, DNPH reagent; Lane B, DNPH derivative of cholesteryl 5-oxovalerate standard; Lane C, DNPH derivative of cholesteryl 9-oxononanoate standard; Lane D, DNPH derivatives of oxidized cholesteryl linoleate; Lane E, DNPH derivatives of oxidized cholesteryl arachidonate; Lane F, DNPH reagent. TLC Band identification: 0, original ester; 1, cholesteryl 12-oxododecenoate; 2, cholesteryl 9-oxononenoate; 3, cholesteryl 5-oxovalerate; 4, 7-ketocholesteryl 12-oxododecenoate; 8, 7-ketocholesteryl 5-oxovalerate. TLC conditions: double development as described under Materials and Methods. SF, solvent front; OR, origin.

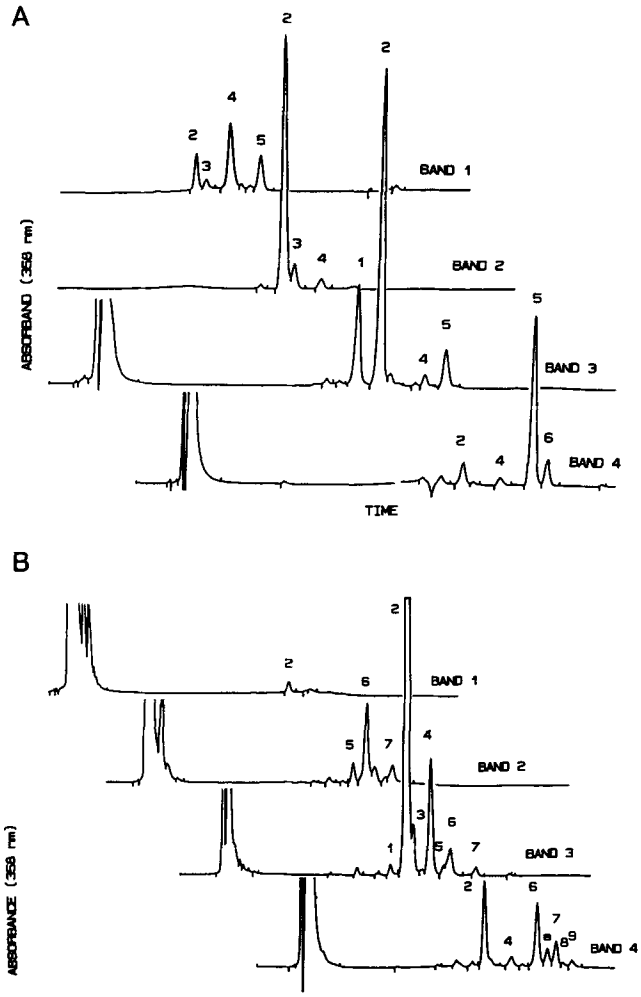

FIG. 5. Reverse-phase high-performance liquid chromatography (HPLC) resolution of dinitrophenylhydrazone derivatives of cholesteryl ester core aldehydes derived from peroxidation of cholesteryl linoleate (upper panel, Bands 1–4) and cholesteryl arachidonate (lower panel, Bands 1–4) following isolation by thin-layer chromatography (Fig. 4). Peak identification (upper panel): 1, cholesteryl 8-oxooctanoate; 2, cholesteryl 9-oxononananoate; 3, cholesteryl 10-oxodecenoate; 4, cholesteryl 11-oxoundecenoate; 5, cholesteryl 12-oxododecenoate; 6, cholesteryl 13-oxotridecadienoate. Peak identification (lower panel): 1, cholesteryl 4-oxobutyrate; 2, cholesteryl 5-oxovalerate; 3, cholesteryl 6-oxocaproate; 4, cholesteryl 7-oxoheptanoate; 5, cholesteryl 9-oxononadienoate; 6, cholesteryl 10-oxodecenoate; 9, cholesteryl 11-oxoundecenoate. HPLC conditions as given under Materials and Methods. Thin-layer chromatography conditions as given in Figure 4.

cholesteryl and oxycholesteryl esters of mainly 5-oxovalerate along with smaller amounts of 4-oxobutyrate, 6-oxohexenoate, 7-oxoheptenoate, 8-oxooctenoate, 9-oxononenoate and others. Band 1 (Fig. 4, Lane E) gave a small peak with a retention time similar to that of the major peak (9-oxononadienoate) in Band 2 (Fig. 4, Lane E). Band 2 (Fig. 4, Lane 4) contained mainly the 5-oxovalerate, along with minor peaks for 6-oxohexenoate, 7-oxoheptenoate, 8-oxooctenoate, 9-oxononadienoate and 10-oxodecadienoate. Band 4 (Fig. 4, Lane E) also contained the 5-oxovalerate of cholesterol on the basis of relative retention time, along with minor peaks corresponding to 7-oxoheptenoate and 9-oxononenoate.

Figure 6A shows the reverse-phase LC/MS profile of the total NCI current of the DNPH derivatives of the core aldehydes derived from the oxidation of cholesteryl linoleate, along with the single ion plots for the molecular weights of the species. These data establish the identities and the relative proportions of the various core aldehydes resolved by the TLC/HPLC combination. Thus, the major components in this fraction are cholesteryl 11-oxoundecenoate (*m/z* 746) and cholesteryl 12-oxododecenoate (*m/z* 780) and cholesteryl 9-oxononanoate (*m/z* 720), as indicated by the ion intensities listed on the left-hand side of the figure. Figure 6B shows the reverse-phase LC/MS total NCI current profile of the DNPH derivatives of the core aldehydes derived from the oxidation of cholesteryl arachidonate along with the molecular ion plots for the species. Here the major components by far are cholesteryl 5-oxovalerate (*m/z* 664) and cholesteryl 7-oxoheptenoate (*m/z* 690), with much smaller amounts of cholesteryl 4-oxobutyrate (*m/z* 650), cholesteryl 6-oxohexenoate (*m/z* 676), 9-oxononadienoate (*m/z* 716) and 10-oxodecadienoate (*m/z* 730), as indicated by the ion intensities listed on the left-hand side of the figure. Again, the LC/MS data establish the identification and proportionation of the core aldehyde species tentatively identified by the TLC/HPLC combination.

The mass spectra of the DPNH derivatives of cholesteryl 9-oxononanoate and cholesteryl 5-oxovalerate isolated from the peroxidation products of cholesteryl linoleate and arachidonate, respectively, corresponded exactly to those of synthetic standards (10). Only single ions corresponding to the molecular weight of these hydrazones were seen in the 500–800 mass range (figures not shown). Figure 7 shows the partial mass spectra of the DNPH derivative of 7-ketocholesteryl 9-oxononanoate (A) and 5,6-epoxycholestanyl 9-oxononanoate (B) recovered from oxidized cholesteryl linoleate and isolated from near the origin of the TLC plate (Fig. 4, Lane D). Again, only single ions corresponding to the molecular weights of the compounds are seen. The minor ion at *m/z* 580 in A is due to the DNPH derivative of 7-ketocholesterol, which overlapped with the 9-oxononanoate on the TLC plate. The minor masses at *m/z* 676 and 772 in B were due to the DNPH derivatives of unidentified components overlapping with the epoxy cholesteryl 9-oxononanoate on the TLC plate.

In addition, we investigated the peroxidation products of the mixed cholesteryl esters of rat plasma by exposing to the *t*-BOOH/$FeSO_4$ system: fresh rat plasma, total lipid extracts of rat plasma and purified cholesteryl esters of rat plasma. In all instances, we were able to demonstrate the formation of ring oxidized cholesteryl esters of palmitic, oleic and linoleic acids and the 5-oxovaleroyl and 9-oxononanoyl esters of cholesterol and oxycholesterol (chromatograms not shown). The various core aldehyde esters were found in proportions reflecting the composition of the fatty acids in the cholesteryl ester mixtures.

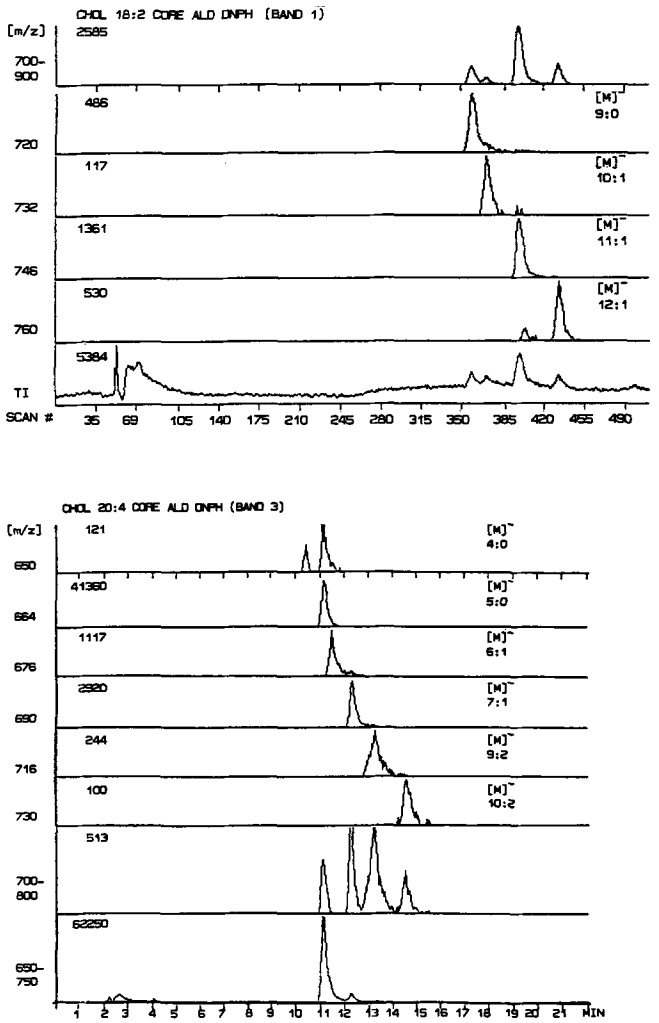

FIG. 6. Reverse-phase liquid chromatography/mass spectrometry (LC/MS) profile of total negative ion current along with molecular ion plots for dinitrophenylhydrazone (DNPH) of core aldehydes derived from oxidized cholesteryl linoleate (upper panel) and oxidized cholesteryl arachidonate (lower panel). Peak identification: as given in the figure (total carbon number:total number of double bonds in the aldehyde moiety). TI, total negative ion current. Other LC/MS conventions as in Figure 3. LC/MS conditions as given under Materials and Methods.

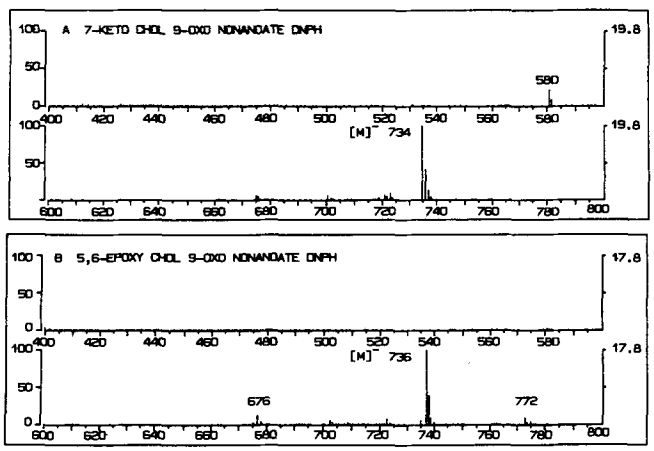

FIG. 7. Partial mass spectra of dinitrophenylhydrazone (DNPH) of 7-ketocholesteryl 9-oxononanoate (A) and DNPH of 5,6-epoxy cholestanyl 9-oxononanoate as obtained by reverse-phase liquid chromatography/mass spectrometry (LC/MS) with negative chemical ionization. LC/MS conditions are as given under Materials and Methods.

Mechanism of oxidation. The mechanism of oxidation of the steryl esters was not specifically investigated in this study, but the choice of the oxidant and the reference to peroxidation requires comment. The term "peroxidation" was adopted in keeping with previous usage. Halliwell and Gutteridge (9) had noted that addition of *tert*-butyl or cumene hydroperoxide along with Fe^{++} is an effective way to stimulate lipid peroxidation *in vitro*. Although free radicals and fatty acid peroxides or hydroperoxides were not identified as indicators of peroxidation in our experiments, conjugated dienes have been detected under comparable reaction conditions (11) suggesting that peroxidation of the polyunsaturated acids may have taken place. Furthermore, the steryl ester core aldehydes identified in this work appear to be of the type proposed for the peroxidation of linoleic and arachidonic acids by Esterbauer *et al.* (22), where a homolytic scission of the two C–C bonds on either side of the hydroperoxy group takes place. It should be noted, however, that the sterol ring oxidation products isolated and identified here are consistent with the mechanism proposed by Kimura and Muto (23) and discussed by Smith (1). These authors have pointed out that *tert*-butyl peroxyl radicals may epoxidize the Δ^5 double bond, and *tert*-butyl oxyl radicals may abstract the C_7 proton to yield C_7 alcohols and C_7 ketones without the generation of peroxides and hydroperoxides as intermediates. It is therefore possible that under the influence of the harsh reagent multiple oxidations may take place by several different pathways.

In summary, the present results demonstrate that polyunsaturated cholesteryl esters are subject to peroxidation in both sterol ring and in the fatty chain, while saturated and monounsaturated fatty acid esters are oxidized only in the ring, when exposed to *t*-BOOH in the presence of ferrous sulfate. The formation of the lipid-soluble core aldehydes from polyunsaturated fatty acid esters of cholesterol has not been previously reported, although it should have been anticipated in view of the known generation of the water-soluble low molecular weight aldehydes. The core aldehydes occur in combination with both unoxidized and oxidized cholesterols, although only the 7-keto cholesterol and the 5,6-epoxycholestanol core aldehydes were actually identified. The esterification of the 3β-hydroxyl group of cholesterol by the saturated or monounsaturated fatty acid had no significant qualitative effect upon the oxidation of the cholesterol ring, although quantitatively it may have provided some protection, as previously reported (1,5,6). The biological activity of the core aldehydes of normal or peroxidized cholesterol is not known. The present study shows that the core aldehyde esters of cholesterol and oxycholesterol are readily hydrolyzed by bacterial cholesteryl ester hydrolase.

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Characteristics of the Thiobarbituric Acid Reactivity of Human Urine as a Possible Consequence of Lipid Peroxidation

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A 532 nm red pigment formed in the thiobarbituric acid (TBA) assay of human urine was characterized after separation of the pigment by high-performance liquid chromatography. The yield of the red pigment was somewhat higher at pH 2 than at pH 5; its development was not inhibited by ethylenediaminetetraacetic acid. The characteristics of the pigment were similar to those of the pigment derived from standard malonaldehyde. The amount of the pigment formed was roughly equal to the content of malonaldehyde derivatives estimated as 1-(2,4-dinitrophenyl)pyrazole. Pigment formation was significantly enhanced by *t*-butyl hydroperoxide (*t*-BuOOH) and ferric ions, which may be due to pigment formed from aldehydes other than malonaldehyde; the presence of these aldehydes was confirmed by the formation of the corresponding 2,4-dinitrophenylhydrazones. The amount of pigment produced from 24-h urine samples of 12 healthy subjects was estimated to be 26–95 nmol/kg, and 65–182 nmol/kg in the presence of *t*-BuOOH. These values are lower than those for urine of rabbit or rat. The TBA reactivity in the absence and presence of *t*-BuOOH of human urine was not related to age or sex. The TBA reactivity of human urine collected in the afternoon and in the evening was higher than that of urine collected in the morning. ***Lipids 28,*** **337–343 (1993).**

Lipid peroxidation has been implicated as being involved in various pathological conditions including aging, carcinogenesis, atherosclerosis, inflammation, ischemia and drug toxicities. Lipid peroxidation products excreted in human urine may thus reflect lipid peroxidation of the whole body. The thiobarbituric acid (TBA) assay (1) has previously been used for the measurement of lipid peroxidation products in rat and in human urine (2–5). Thus, heating of urine with TBA in acidic medium has been shown to give a red malonaldehyde TBA (1:2) adduct with a maximum absorption at 532 nm (6).

Although the red pigment had been assumed to be specific for malonaldehyde, other aldehydes generated during lipid peroxidation have been shown to produce the same pigment (1,7–14). Pigment formation from malonaldehyde is quantitative and is little affected by pH, added organic hydroperoxides or ferric ion. In contrast, pigment formation from alkadienals and alkenals is not quantitative, is highly pH-dependent (12,13) and is greatly enhanced by added organic hydroperoxides (8–14) and ferric ions (13,14). Our previous studies have shown that the pigment formed from oxidized fats and oils is due to alkadienals and/or alkenals (11,13), and that the pigment formed from rat liver and brain homogenate is due to both alkadienals and malonaldehyde (12,14).

In the present study, we characterized the red pigment formed in the TBA assay of human urine and determined which aldehydes generated during lipid peroxidation are responsible for pigment formation. We also determined the amounts of pigment formed from urine of healthy human subjects.

MATERIALS AND METHODS

Materials. TBA, 2,4-dinitrophenylhydrazine (DNPH), propanal, butanal, hexanal and 2,4-heptadienal were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2-Butenal, 2-hexenal, 2,4-hexadienal and tetramethoxypropane (TMP) were obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan). 2-Pentenal and 2-heptenal were obtained from Aldrich Chemical Company (Milwaukee, WI). *t*-Butyl hydroperoxide (*t*-BuOOH) (70% in water) was obtained from Sigma Chemical Company (St. Louis, MO); its concentration was determined to be 7.0 M by iodometric titration. Butylated hydroxytoluene (BHT) was obtained from Nikki Universal Company (Tokyo, Japan). *N*-(2-propenal)aminoacetic acid was prepared as described (15). Glacial acetic acid was a special reagent grade product of Wako Pure Chemical Industries.

Analysis. Absorption spectra were measured on a Hitachi U-2000 (Tokyo, Japan) or a Shimadzu UV-240-Visible (Osaka, Japan) spectrophotometer. High-performance liquid chromatography (HPLC) was carried out on a Hitachi L-600 or L-655-A11 liquid chromatograph equipped with a column (4.6 mm i.d. × 250 mm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). The fractions were detected with a Hitachi L-4200 UV-VIS or a Shimadzu SPD-6A detector.

Urine. Urine was collected from healthy human male and female subjects, Japanese White male rabbits and Wistar male rats and used for analysis within several hours. For determination of one-day amounts of the red pigment, 24-h urine was pooled for the assay.

Major TBA-reactive substances of human urine. The TBA-reactive substances of human urine were partially purified through an anion exchange column and by gel filtration according to the method of Hadley and Draper (16). Human urine (250 mL) was applied to a column (3 cm i.d. × 22 cm) of AG1-X8 (chloride form), the column was eluted with 1 L of linear gradient sodium chloride solution (0–1 M), and 5-mL fractions were collected. Most of the color of urine due to urobilin, and about 30% of the TBA-reactive substances were eluted in fractions (20–60) (200 mL) (shown later in Fig. 2). About 70 mL of the fractions were condensed into 6 mL and passed through a column (3 cm i.d. × 21 cm) of Sephadex G-10 equilibrated with water, and the column was eluted with 100 mL of water. The TBA-reactive substances loaded were completely eluted, and the eluate was condensed to 10 mL.

TBA assay. All TBA assays were performed by the two-step mode to obtain reproducible data for alkadienals (11).

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Abbreviations: BHT, butylated hydroxytoluene; DNPH, 2,4-dinitrophenylhydrazine; DNPP, 1-(2,4-dinitrophenyl)pyrazole; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TBA, thiobarbituric acid; *t*-BuOOH, *tert*-butyl hydroperoxide; TMP, tetramethoxypropane.

The assays were performed with addition of BHT to avoid undesirable peroxidation during the assays (13,14).

Method A. Two mL of a solution containing 20 nmol TMP or urine (human urine 2.0 mL, rabbit and rat urine 0.5 mL) in water, 0.10 mL of 0.5% BHT solution in glacial acetic acid (the final concentration of acetic acid was 2%) and 3.0 mL of 0.5% TBA solution in water were placed in this order into a screw-cap test tube. For studying the effect of *t*-BuOOH, 10 μL of a 0.5–2 M *t*-BuOOH solution in acetic acid was added to the mixture. For studying the effect of ferric ion and ethylenediaminetetraacetic acid (EDTA), 0.5% TBA solution was replaced by 0.5% TBA/0.2–4 mM $FeCl_3$ (or EDTA disodium salt) in water. The mixture was kept at 5°C for 60 min and then was heated at 100°C for 20 min. After cooling, the mixture was extracted with 3 mL of chloroform and then centrifuged at 650 × *g* for 10 min. Absorbance at 532 nm of the aqueous phase was determined, and the amount of red pigment was calculated on the basis of the absorbance and the molecular extinction coefficient (156000) of red pigment (17). The aqueous phase was subjected to HPLC, and the column was eluted with 0.04 M acetate buffer (pH 5.5)/methanol (6:4 vol/vol) at a flow rate of 0.8 mL/min. A peak was detected at 532 nm. Red pigment from a standard TMP solution appeared at a retention time of 8.0 min. The amount of red pigment from urine was determined by comparing the peak area of red pigment with that of the standard TMP solution. The amounts of red pigment from human urine linearly increased with the volume up to 2.0 mL, and those from rabbit and rat urine linearly increased with the volume up to 0.5 mL.

Method B. Method B is a modification of the method of Ohkawa *et al.* (18). Two mL of a solution containing 20 nmol TMP in water or 2.0 mL of human urine, 1.50 mL of 20% acetic acid adjusted at the indicated pH value by addition of 10 N NaOH, 50 μL of 1% BHT solution in glacial acetic acid and 1.50 mL of 0.8% TBA solution in water were placed in this order into a test tube. For studying the effect of *t*-BuOOH, 10 μL of 1 M *t*-BuOOH solution in acetic acid was added. For studying the effect of ferric ions and EDTA, 0.1 mL of 100 mM $FeCl_3$ or EDTA disodium salt solution in water was added. The mixture was kept at 5°C for 60 min and was then heated at 100°C for 60 min. After cooling, the mixture was extracted with 3 mL of chloroform and centrifuged. The aqueous phase was subjected to HPLC as described.

Determination of malonaldehyde as DNPH derivative. Malonaldehyde was determined as 1-(2,4-dinitrophenyl)pyrazole (DNPP) by reaction with DNPH according to the established method (19,20) with slight modifications (14). Reference standard DNPP was prepared as described (14). Thus, a mixture of 1.5 mL of urine or an aqueous solution containing 30 nmol TMP, along with 1.2 mL of 0.25% (wt/vol) DNPH solution in 1 N HCl, was heated at 100°C for 30 min. The reaction mixture was extracted with chloroform as described (14), and the extract was subjected to HPLC. The column was eluted with acetonitrile/0.01 N HCl (45:55, vol/vol) at a flow rate of 1.5 mL/min. The peak due to DNPP appeared at a retention time of 7.0 min and was detected at 300 nm. Malonaldehyde content of urine was determined by comparing the peak height of DNPP from urine with that of DNPP from the TMP standard solution.

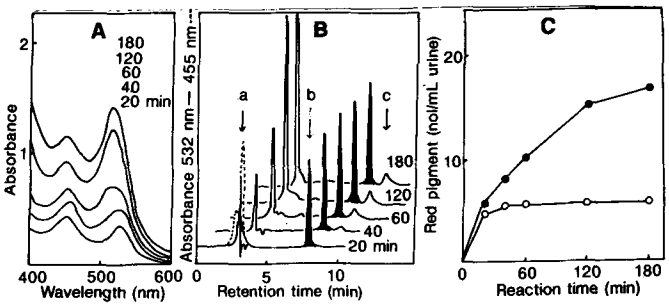

FIG. 1. Profiles of pigment formation in the thiobarbituric acid (TBA) assay of human urine. A: Absorption spectra of the TBA reaction mixture (method A) of human urine (2 mL) heated at 100°C for the indicated period. The reaction mixture of standard tetramethoxypropane (TMP) heated for 20–180 min showed the same absorbance with a single absorption maximum at 532 nm. B: High-performance liquid chromatography (HPLC) pattern of the TBA reaction mixture (method A) of human urine heated at 100°C for the indicated period. Three peaks, a, b and c, were detected. Red pigment (532 nm) from standard TMP eluted at a retention time of 8.0 min (peak b). C: Time course of 532 nm red pigment determined by absorbance (A) (●), and that determined by peak b in HPLC analysis (B) (○). The control reaction mixture without TMP and urine showed no significant coloration and HPLC peaks.

Determination of aldehydes with DNPH. Aldehydes other than malonaldehyde were determined as previously described (11). DNPH (50 mg) was dissolved in 100 mL of 1 N HCl, and the solution was extracted twice with 50 mL of *n*-hexane to remove impurities. Two mL of urine or an aqueous solution containing 5 μmol standard aldehyde was added to 2.0 mL of the solution. The mixture was vigorously shaken at room temperature for 5 min in the dark and extracted six times with 3 mL of benzene. The organic layers were combined and evaporated to dryness, and the residue was redissolved into 0.5 mL of benzene. The solution was applied to a column (1 cm i.d. × 3.5 cm) of silica gel, and the column was eluted with benzene. The hydrazone fractions (3–8 mL) and DNPH fractions (15–32 mL) were separated, the former being evaporated to dryness to be redissolved into 5.0 mL of methanol. Absorption spectra of the solution gave maxima at 360 nm. The amounts of aldehyde were calculated on the basis of the average molar extinction coefficient (26000) of the various hydrazones at 370 nm (21). The methanolic solution was evaporated to dryness and dissolved into 0.2 mL of acetonitrile for HPLC analysis. The column was eluted with acetonitrile/water (3:2, vol/vol) at a flow rate of 1.5 mL/min (14). The peaks were detected at 370 nm (shown later in Fig. 5).

RESULTS

The TBA reaction of the reference standard TMP and human urine was carried out by heating at 100°C for 20 min in 2% acetic acid containing 0.01% BHT (method A). While TMP produced 532 nm red malonaldehyde TBA (1:2) adduct quantitatively, human urine produced pigments with two absorption maxima at 455 and 532 nm (Fig. 1A). The absorbance was gradually increased by prolonged heating up to 180 min, and the maximum at 532 nm was shifted to 518 nm. The 532 nm red pigment could not be accurately determined by direct spectrophotometry

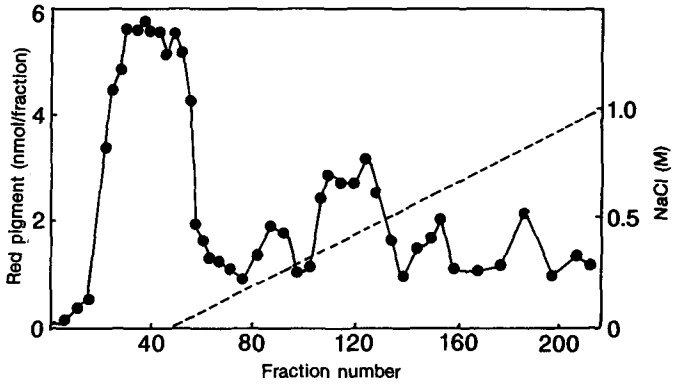

FIG. 2. Anion exchange column chromatography of the thiobarbituric acid (TBA)-reactive substance of human urine. Human urine (250 mL) containing the substances to produce 660 nmol 532 nm red pigment was applied to an AG1-X8 column (3 × 22 cm) with sodium chloride linear gradient elution, and 5-mL fractions were collected. The TBA-reactive substances were detected in the assay by their absorbance at 532 nm (method A).

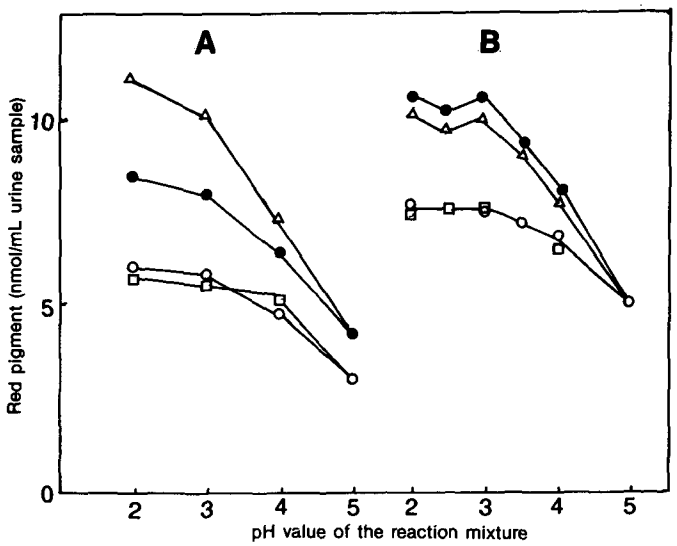

FIG. 3. Effect of pH, *t*-BuOOH, ferric ions and EDTA on the development of the red pigment in the TBA assay of human urine. Human urine (A) and the major TBA-reactive substances obtained by anion exchange column chromatography (see Fig. 2) (B) were subjected to the TBA assay (method B) in the absence (○) or presence of 2 mM *t*-BuOOH (●), 2 mM ferric ions (△) and 2 mM EDTA (□). The reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figure 1. *t*-BuOOH, *tert*-butyl hydroperoxide; EDTA, ethylenediaminetetraacetic acid.

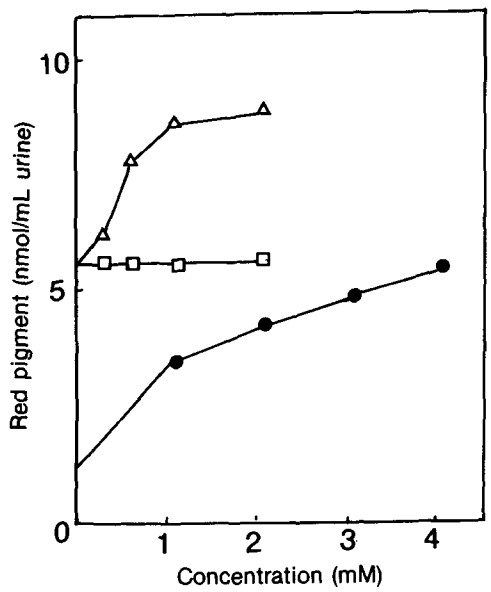

FIG. 4. Concentration-dependent effect of *t*-BuOOH, ferric ions and EDTA on the red pigment development in the TBA assay of human urine. Urine samples were subjected to the TBA assay (method A) in the presence of *t*-BuOOH (●), ferric ion (△) and EDTA (□) at the indicated final concentration. The reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figures 1 and 3.

owing to the production of the interfering pigments. In HPLC, the 532 nm red pigment was separated from the interfering pigments (peak a and peak c) and eluted at a retention time of 8.0 min (peak b) (Fig. 1B). The absorption spectrum of peak b showed a single maximum at 532 nm of red malonaldehyde TBA (1:2) adduct. The amount of the red pigment determined in peak b was constant during the heating periods from 20 to 180 min (Fig. 1C). The amount of the red pigment produced during 20 min as estimated by direct spectrophotometry was 20–30% higher than that measured by HPLC. Hence, HPLC separation was necessary for accurate quantitation of the red pigment from urine.

When human urine was dialyzed against water, about 90% of the TBA-reactive substances that produce the red pigment were removed (data not shown), indicating that most of the TBA-reactive substances were low molecular weight compounds. When human urine was passed through an anion exchange column according to the method of Hadley and Draper (16), the TBA reactive-substances were resolved into several peaks (Fig. 2). The chromatographic profile was similar to that of rat urine (16), and the TBA-reactive substances of human urine were not single. Major TBA-reactive substances (about 30%) of human urine were eluted in the first fraction. When the red pigment from human urine was determined by the methods of Ohkawa *et al.* (18), Buege and Aust (22) and Uchiyama and Mihara (23), similar results were obtained as long as the pigment was determined after HPLC separation (data not shown).

The effect of the pH of the TBA reaction mixture (method B) on the red pigment development from human urine (Fig. 3A) and from the major TBA-reactive substances separated by anion exchange column chromatography (Fig. 3B) was further investigated (open circles). Pigment formation was somewhat dependent on the pH value of the reaction mixture; it was maximal at pH 2, and 1.5-fold higher than at pH 5. Addition of 2 mM *t*-BuOOH (Fig. 3; closed circles) or ferric ions (Fig. 3; open triangles) increased pigment development in the pH ranges between 2 and 5, suggesting the presence of alkadienals and/or alkenals whose reactivity is known to be enhanced by these reagents (8–14). Addition of 2 mM EDTA (Fig. 3; open squares) did not affect the pigment development throughout the pH ranges, suggesting that a trace amount of contaminated ferric and ferrous ions had been already chelated and inactivated in the reaction mixtures containing urine samples so as not to enhance the TBA reactivity due to alkadienals and alkenals. Figure 4 shows

TABLE 1

Comparison of the Amount of Red Pigment Produced in the Thiobarbituric Acid (TBA) Assay of Human Urine Containing Malonaldehyde Derivatives and Other Aldehydes

Urine sample	532 nm Red pigment[a] (nmol/mL urine)			Malonaldehyde derivatives[b] (nmol/mL urine)	Other aldehydes[c] (nmol/mL urine)
	+ None	+ *t*-BuOOH	+ $FeCl_3$		
Urine					
1	10.6			15.3	
2	8.0			6.5	
3	11.0			19.5	
4	7.0			5.0	
5	4.3	6.1	7.9		11.5
6	3.9	5.7	8.3		9.1
7	4.7	7.6	8.3		9.0
8	2.2	3.6	5.0		5.4
Major TBA-reactive substances of urine[d]					
9	19.2	20.9	24.1	24.2	24.2

[a]Red pigment (532 nm) was determined by high-performance liquid chromatography of the TBA reaction mixture (method A) heated at 100°C for 20 min in the absence or presence of 2 mM *tert*-butyl hydroperoxide (*t*-BuOOH) or $FeCl_3$.

[b]Malonaldehyde content was determined as 1-(2,4-dinitrophenyl)pyrazole by 2,4-dinitrophenylhydrazine (DNPH) method.

[c]Other aldehyde content was determined as hydrazones of DNPH by silica gel column chromatography.

[d]The major TBA-reactive substances were obtained by anion exchange column chromatography (see Fig. 2).

concentration-dependent enhancement of the red pigment development by *t*-BuOOH and ferric ions in the TBA assay (method A) of human urine samples. The pigment development from malonaldehyde derivatives, TMP and *N*-(2-propenal)aminoacetic acid was not enhanced by 2 mM ferric ions and not inhibited by 2 mM EDTA under the same conditions (data not shown).

The amounts of red pigment formed from human urine (Table 1, samples 1–4) and the major TBA-reactive substances obtained by anion exchange column chromatography (Table 1, sample 9) were compared with the contents of malonaldehyde derivatives determined as DNPP by the DNPH method (19,20). The amounts of red pigment obtained without added *t*-BuOOH and ferric ions were roughly equal to or even lower than the levels of malonaldehyde derivatives measured. Hence, the TBA reactivity of human urine without added *t*-BuOOH and ferric ions was ascribed to malonaldehyde derivatives.

The enhanced amounts of the red pigment that developed in the presence of *t*-BuOOH or ferric ions were compared with the levels of other aldehydes determined as the hydrazones of DNPH (Table 1, samples 5–8). The amounts of other aldehydes were high enough to account for the enhanced pigment formation. When the hydrazones from the major TBA-reactive substances obtained by anion exchange column chromatography (Table 1, sample 9) were analyzed by HPLC (Fig. 5), several peaks due to the hydrazones of aldehydes were detected. One of the hydrazones was identified as the hydrazone of 2,4-hexadienal by cochromatography with an authentic standard (data not shown). Hence, the red pigment development from urine enhanced by *t*-BuOOH and ferric ions may be due to alkadienals and/or alkenals.

One-day amounts of the red pigment from 24-h urine of 12 healthy subjects were determined and compared (Table 2, lane 1). The amounts of the pigment were 26–95 nmol/kg · day in the absence of *t*-BuOOH and 65–182 nmol/kg · day in the presence of *t*-BuOOH. All the subjects excreted both malonaldehyde derivatives and other aldehydes as TBA-reactive substances. However, the amount of pigment formed varied from subject to subject and also varied by the day of collection of urine from a specific subject. The TBA reactivity of urine with and without added *t*-BuOOH was not related to age or sex. One-day amounts of the pigment from rabbit urine (Table 2, lane 2) and rat urine (Table 2, lane 3) were similarly

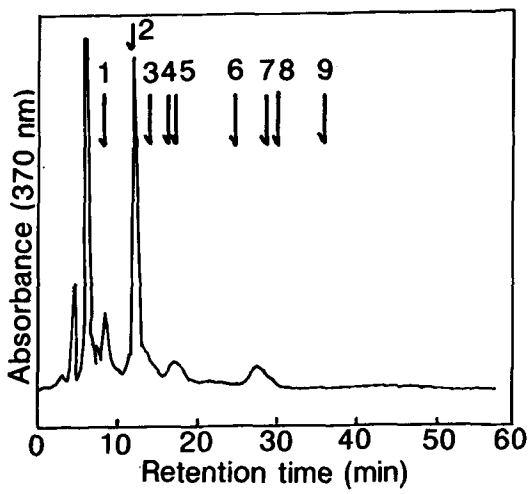

FIG. 5. HPLC chromatogram of the hydrazone fraction of silica gel column chromatography from the major TBA-reactive substances obtained by anion exchange column chromatography (see Fig. 2 and Table 1, lane 9). Hydrazones of standard aldehydes were eluted at the retention times indicated by arrows; 1, propanal; 2, butenal; 3, butanal; 4, pentenal; 5, hexadienal; 6, hexenal; 7, heptadienal; 8, hexanal; and 9, heptenal. The control reaction mixture without sample revealed no hydrazone fraction in silica gel column chromatography and no peaks in HPLC. Abbreviations as in Figure 1.

TABLE 2

One-Day Amounts of Red Pigment Produced in the Thiobarbituric Acid (TBA) Assay of Urine of Human, Rabbit and Rat[a]

Subject			One-day 532 nm red pigment (nmol ± SD/kg•day)		
Sex	Age (year)	Body weight (kg)	Number of determinations	+ None	+ *t*-BuOOH
Human male	22	57	4	44 ± 7	92 ± 13
	23	54	1	54	65
	24	58	1	87	107
	25	58	1	87	107
	34	65	4	30 ± 4	76 ± 13
	71	53	1	92	119
Human female	18	51	1	60	90
	18	55	1	88	106
	46	44	4	84 ± 11	156 ± 26
	49	50	1	69	110
	50	58	5	73 ± 10	117 ± 18
	85	36	1	76	87
Rabbit male	1	3.25	4	616 ± 121	1932 ± 338
	1	3.20	4	445 ± 83	1415 ± 269
	1	3.15	4	524 ± 163	1527 ± 430
	2	4.30	7	363 ± 117	817 ± 261
	3	4.45	5	312 ± 92	733 ± 255
Rat male	0.13	0.18	2	88	578
	0.13	0.23	2	148	860
	0.13	0.21	2	195	354
	0.13	0.22	2	192	703
	0.13	0.20	2	155	849
	1	0.44	4	125 ± 30	1079 ± 308
	1	0.42	4	112 ± 24	868 ± 147
	1	0.38	4	224 ± 16	1017 ± 154
	1	0.44	4	122 ± 30	911 ± 182
	3	0.43	6	63 ± 38	370 ± 139
	3	0.40	2	55	267
	3	0.35	6	57 ± 31	371 ± 230

[a]24-h Urine was collected and subjected to the TBA assay (method A) heated at 100°C for 20 min in the absence or presence of 2 mM *t*-BuOOH. The red pigment was determined by high-performance liquid chromatography. The one-day amount of pigment (nmol ± SD/kg•day) was corrected for the volume of urine and the body weight. *t*-BuOOH, *tert*-butyl hydroperoxide.

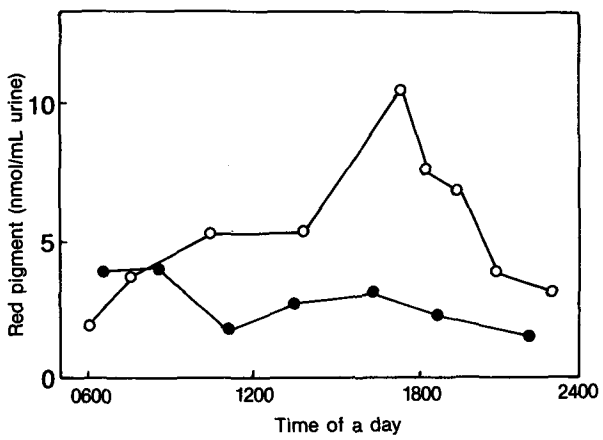

FIG. 6. The TBA reactivity of human urine collected at different times throughout a day. Two human urine samples were subjected to the TBA assay (method A), and the reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figure 1.

determined. The TBA reactivity of urine of these animals was much higher; thus humans excreted lower amounts of malonaldehyde derivatives and other aldehydes than did the animals.

Urine was collected from healthy human subjects at different times of the day, and the amounts of the pigment produced without *t*-BuOOH were determined and compared (Fig. 6). It was found that the TBA reactivity of urine collected in the afternoon or in the evening was higher than that collected in the morning.

DISCUSSION

The TBA reaction of human urine produced at least three pigments, namely 455 nm yellow, 532 nm red and 518 nm orange pigments, as shown in Figure 1. The yellow pigment may be derived from alkanals (24,25) and/or sugars (26) and cannot be regarded as an index of lipid peroxidation. The red pigment is only the pigment that appears to reflect lipid peroxidation. The orange pigment was produced when the reaction mixture was heated for prolonged periods. Bidder and Sipka (27) have demonstrated that the absorbance at 532 nm of the TBA reaction mixture of urine progressively increased with heating time, and they suggested that the abnormal increase is due to materials other than malonaldehyde derivatives. As alkadienals and alkenals produce 532 nm red pigment in a time course similar to that of malonaldehyde derivatives (7,8), the abnormal increase may be due to the development of

chromogens other than 532 nm red pigment. Several substances in urine have been shown to produce these different chromogens (1,28–31). HPLC separation of the pigment is necessary for accurate quantitation of the red pigment that appears to reflect lipid peroxidation products in urine.

The TBA reactivity of urine that produces the red pigment is barely dependent on the method used, whereas the reactivity of fats and oils (13) and rat liver and brain homogenate (12,14) have been shown to be greatly dependent on the method used. The yield of the red pigment from urine was somewhat higher at pH 2 than at pH 5, and its development was not inhibited by EDTA. The characteristics of the pigment development were similar to those of standard malonaldehyde derivatives. The amount of red pigment formed was roughly equal to the content of malonaldehyde derivatives estimated as DNPP. The TBA reactivity of urine thus appears to be due to malonaldehyde derivatives. However, the pigment development enhanced by *t*-BuOOH and ferric ions seem to be due to aldehydes other than malonaldehyde derivatives (13,14), whose presence was confirmed by hydrazone formation with DNPH.

The present results are consistent with earlier observations. Draper *et al.* (2–4) had demonstrated that the TBA reactivity of rat urine increased when supplemented with malonaldehyde derivatives, a vitamin E-deficient diet, iron or carbon tetrachloride, and that the TBA reactivity of human morning urine increased by consuming a supplement of n-3 fatty acids (5). They have identified malonaldehyde derivatives, such as *N*α-acetyl-ε-(2-propenal)lysine (32), *N*-(2-propenal)serine (16), *N*-ε-(2-propenal)lysine (33), *N*-(2-propenal)ethanolamine (34) and guanine-malonaldehyde adduct (35) as rat urinary metabolites. Ekstrom *et al.* (19,36) demonstrated that administration of chloroform or hydroquinone to rats resulted in high excretion of malonaldehyde derivatives as estimated by the DNPH method. Our results give additional evidence for the excretion of malonaldehyde derivatives in urine. Lee *et al.* (37) have recently demonstrated that HPLC separation of rat urine gives six TBA-reactive substances, including aldehydes other than malonaldehyde derivatives. Our results on the TBA reactivity of human unseparated urine in the presence of *t*-BuOOH or ferric ions clearly show the presence of aldehydes other than malonaldehyde derivatives.

It has been shown that the TBA reactivity of oxidized fats and oils is due to alkadienals and/or alkenals (11,13,14), and that the reactivity of tissue homogenate is mainly due to alkadienals and, to a lesser extent, to malonaldehyde derivatives (12,14). By contrast, the TBA reactivity of urine was found to be due mainly to malonaldehyde derivatives and, to a lesser extent, to other aldehydes only when the reaction was carried out in the presence of *t*-BuOOH or ferric ions. It is interesting to note that the TBA-reactive substances of urine were different from those of tissues. Nonpolar TBA-reactive substances composed of alkadienals generated in tissues may stay longer in tissues, and the polar TBA-reactive substances composed of malonaldehyde derivatives may be more readily excreted in urine.

The amounts of red pigment produced from 24-h urine of 12 healthy subjects were estimated to be 26–95 nmol/kg, and 65–182 nmol/kg in the presence of *t*-BuOOH, the values being lower than those from urine of rabbit or rat. Excretion of malonaldehyde derivatives and other aldehydes had no relationship to the age or sex of the subjects. TBA reactivity due to malonaldehyde derivatives was higher in the afternoon or in the evening than in the morning, suggesting that the physical activity of a subject during daytime may contribute to increased excretion. It has also been suggested that intake of polyunsaturated fatty acids leads to higher excretion of malonaldehyde derivatives in urine (5).

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Fatty Acids in the Lipids of *Drosophila* Heads: Effects of Visual Mutants, Carotenoid Deprivation and Dietary Fatty Acids

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Lipids of *Drosophila* heads were extracted and separated by high-performance thin-layer chromatography. Fatty acid compositions of major phospholipids as well as of triglycerides were analyzed by gas-liquid chromatography. Proportions of the major fatty acids (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3) varied depending on the lipid analyzed. Docosahexaenoic acid (22:6), common in vertebrate photoreceptors and brain, and arachidonic acid (20:4), a precursor of eicosanoids, were lacking. A comparison of the fatty acid composition of the diet *vs.* the head suggested that *Drosophila* can desaturate but may not be able to elongate fatty acid carbon chains. Fatty acid analyses were carried out after the following visual system alterations: i) the transduction mutant where *no receptor potential* results from a deficit in phospholipase C; ii) an allele of *eyes absent*; iii) the mutant outer rhabdomeres absent which lacks visual pigment and rhabdomeres in the predominant type of compound eye receptor, rhabdomeres 1 through 6; and iv) carotenoid deprivation which reduces opsin and rhabdomere size. We also evaluated aging by comparing newly-emerged *vs.* aged wild-type flies. Alterations in fatty acid composition based on some of these manipulations were found. Based on comparisons between flies reared on media differing in C_{16} and C_{18}, there is an indication that diet readily affects tissue fatty acid composition.
***Lipids* 28, 345–350 (1993).**

In previous studies (1,2) we have quantified the fatty acids of phospholipids (PL) and triglycerides (TG) of *Drosophila* heads. Our primary interest in the fatty acids is based in their importance as structural components of the PL of visual membranes, which, in the fly, are tightly packed microvillar organelles called rhabdomeres. In addition, fatty acids are of interest in such membranes, whose main function is signal transduction, since they can serve as precursors of signalling molecules.

The strategy of genetic dissection (3), comparing strains with *vs.* without identified visual structures or processes in *Drosophila*, helped us to dissect the fatty acids of the visual system. In the present study, we analyzed: i) outer rhabdomeres absent *(ora)* an opsin mutant which selectively eliminates the predominant rhabdomeres, 1 through 6, (R1–6) of the compound eye (3); ii) *eyes absent (eya)* which eliminates the compound eyes and reduces the optic lobes; and iii) *no* receptor potential *(norpA)* whose phenotype results from a phospholipase C (PLC) deficiency (4,5). We also utilized carotenoid deprivation which lowers opsin in all receptors (6) and makes the rhabdomeres smaller (7). Moreover, we compared samples from aged *vs.* control heads and samples derived from animals reared on diets with different fatty acid compositions.

MATERIALS AND METHODS

Animals. Stocks of *Drosophila melanogaster* were raised at room temperature under cyclic lighting on a standard diet known to be adequate for visual receptor development (8). In the medium of agar, brewer's yeast, molasses and corn meal, the compounds most relevant to visual development were the yellow pigment (zeaxanthin) in corn meal and a supplement of β-carotene (0.13 mg/mL), both precursors of rhodopsin's chromophore. Flies were deprived of carotenoids by rearing on Sang's medium (9); replete controls were raised on Sang's medium supplemented with β-carotene at 0.13 mg/mL. Importantly, the diets differed in fatty acid composition (Table 1).

Wild type (Oregon-R) was used as the normal control fly. Our *norpA* stock was $norpA^{EE5}$, an effective allele (10), originally from Prof. Seymour Benzer's laboratory at the California Institute of Technology (Pasadena, CA). An allele of *eyes absent (eya)* was obtained from Prof. Terry McGuire at Rutgers University (New Brunswick, NJ); these flies lack the compound eyes but not the simple eyes (ocelli) (11). We used the well-studied outer rhabdomeres absent *(ora)* mutant allele of the R1–6 opsin gene in which the outer rhabdomeres (R1–6) are selectively deleted (12); R1–6 rhabdomeres comprise the predominant photoreceptive organelle in the compound eye. For *ora*, as well as the wild type controls in the *ora* experiments, flies were aged in cyclic lighting one week or more because this manipulation insured a better deletion of *ora*'s rhabdomeres (12). For all other types, flies were newly-eclosed, that is, newly emerged from the pupa case.

TABLE 1

Percentages of Fatty Acids in Medium

Fatty acid	Regular diet (mole %)	Sang's diet (mol %)
16:0	74.37	61.13
18:0	13.61	20.49
18:1	10.06	18.38
18:2	1.96	trace[a]
18:3	trace	trace

[a]Less than 1%.

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Abbreviations: DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; *eya*, eyes absent; GLC, gas-liquid chromatography; HPTLC, high-performance thin-layer chromatography; *norpA*, *no* receptor potential; *ora*, outer rhabdomeres absent; PC, phosphatidylcholine; PC_r, a likely PC analogue migrating to the right of PC in HPTLC; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PLC, phospholipase C; PS, phosphatidylserine; R1–6, rhabdomeres 1 through 6; TG, triglyceride; UV, ultraviolet.

Extractions. Glass vials with flies were plunged into liquid nitrogen. When flies were frozen, shaking vials vigorously separated heads from bodies, and 100 heads were sorted quickly with a brush at room temperature. Heads were then put into (usually) 1 mL of ice cold 0.32 M sucrose with 50 mM Tris-HCl buffer (pH = 7.4) and ethylenediaminetetraacetic acid (EDTA) (1 mM) and homogenized with a tightly fitted Teflon pestle. Then, 4 vols of chloroform/methanol (2:1, vol/vol) was added, followed by vortexing. In order to facilitate the recovery of the anionic PL, 50 μL of 40 mM ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) was added to the tube, followed by revortexing. The tubes were centrifuged at 1500 × *g* for 5 min, and the organic phase was recovered and passed through a mini-column containing anhydrous Na_2SO_4. For more complete extraction of the acidic PL, a second extraction with 2 vol of chloroform/methanol (2:1, vol/vol) with 0.4% HCl was performed. The second organic extract was neutralized with one drop of 4 N NH_4OH before combining with the first one, and the extract was then evaporated to dryness.

High-performance thin-layer chromatography (HPTLC) and gas-liquid chromatography (GLC). The lipids were resuspended in chloroform/methanol (2:1, vol/vol). Three separate aliquots of the samples were spotted onto 10 × 10 cm HPTLC plates (Silica gel, 60, E. Merck, Darmstadt, Germany). These plates had previously been dipped into a solution containing 1% potassium oxalate with 2 mM EDTA and then diluted with methanol in the ratio of 3:2 (vol/vol). Data from the three batches were averaged, and standard deviations were calculated.

The procedures for HPTLC separation of PL have been described (13). Briefly, plates were developed in the first solvent system containing chloroform/methanol/acetone/29% ammonium hydroxide (70:40:10:10, by vol). After drying, plates were exposed to HCl vapor for 3 min for hydrolysis of the alkenyl ether linkages and were further developed in a second dimension using a solvent system containing chloroform/methanol/acetone/acetic acid/0.1 M ammonium acetate (70:30:27.5:2.25:5, by vol). After development, the plates were sprayed with 2,7-dichlorofluorescein, and the lipid spots were identified under a ultraviolet (UV) lamp. Individual PL or TG (together with silica gel) were scraped into test tubes. Prior to transmethylation, a known amount of heptadecanoic acid (17:0) methyl ester was added to each sample as an internal standard and for quantification of the PL in the sample.

Conditions for the analysis have been described previously (13). Briefly, conversion of the glycerolipid acyl groups to their methyl esters was achieved by base-catalyzed methanolysis. We added 2 mL of 0.5 M NaOH in anhydrous methanol (sodium methoxide) to the sample. After incubation at room temperature for 10 min, the mixture was partitioned into two phases by adding 4 mL of chloroform and 1.5 mL of water. After phase separation, the organic layer was removed and filtered through a mini-column of anhydrous Na_2SO_4. The organic solvent was evaporated to dryness under nitrogen, and the fatty acid methyl esters were redissolved in hexane. Fatty acid methyl esters were separated using a Supelco (Bellefonte, PA) SP2330 0.2 μm capillary column, 30 m long and 0.25 mm inside diameter, with helium as the carrier gas. Column temperature was programmed from 140 to 195°C at 4°C/min. Analysis was with a HP 5890 gas-liquid chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame-ionization detector. Recoveries of fatty acids from phospholipids after methanolysis had been verified to be over 95% (13).

RESULTS

Experiments carried out in this study involved *Drosophila* that were reared either on a diet based on natural products such as yellow cornmeal, molasses and yeast, the regular diet (8), or a more defined medium which was useful for deprivation or supplementation with vitamin A, the Sang's diet (9). Importantly, the diets differed in fatty acid composition as shown in Table 1. For example, the Sang's diet had a considerably higher proportion of 18:1 and a slightly lower proportion of 18:2 as compared with the regular diet.

Figure 1 shows a typical GLC tracing depicting the fatty acids from phosphatidylethanolamine (PE) in *eya* together with the internal standard (17:0). The most conspicuous finding was the absence of polyunsaturated fatty acids with chain lengths longer than 18 carbons, an observation in good agreement with that reported by Yoshioka *et al.* (14). Despite the presence in the diets of trace amounts of the essential fatty acids with 18:2 and 18:3 acyl chains, substantial proportions of these fatty acids are present in the PL of *Drosophila* heads. Since the fatty acids of *Drosophila* heads showed a substantial proportion of 16:1 which was not found in either food, it is concluded that flies are capable of desaturating 16:0. When we omitted the 17:0 standard, our GLC tracings did not reveal detectable levels of odd-numbered fatty acids, which is in disagreement with the data of Yoshioka *et al.* (14).

During the analysis of PL and TG by GC, it is possible to quantify the mass of the lipids obtained. The relative

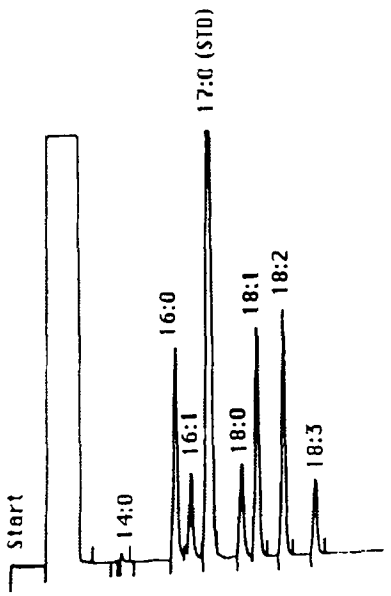

FIG. 1. A typical gas-liquid chromatogram, in this case from the *eyes absent* mutant for the phospholipid phosphatidylethanolamine. Fatty acid peaks at 14:0, 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 are labeled. The high peak at 17:0 is due to the standard (STD); when the STD is omitted (data not shown), there is virtually no reading at this location.

recovery of the standard (17:0 methyl ester), which had been added in known amount to samples from specified numbers of fly heads, should be inversely proportional to the quantity of each lipid in each strain. Data in Table 2 are calculated to represent the amount of each specified lipid for each experimental fly type studied (*eya*, *ora*, *norpA* and vitamin A deprived). PE, phosphatidylcholine (PC), a lipid which migrates to the right of PC on the HPTLC plate (PC_r), phosphatidylinositol (PI), phosphatidylserine (PS) and TG were analyzed. We divided the recovery of standard (averaged from the three samples partitioned from each 100-head batch) from each appropriate control strain by the recovery for each experimental strain. Lipid extracts were prepared with *eya* and *norpA* (*vs.* newly-eclosed wild-type controls), *ora* (aged to insure rhabdomere loss *vs.* aged wild-type controls) and carotenoid deprived and replete (control) wild-type reared on the same food (Sang's medium without *vs.* supplemented with β-carotene, respectively). As expected, *eya* shows the largest decrease in lipid content since the mutation eliminates the compound eyes entirely and reduces the optic lobes. Also, as expected, vitamin A deprived flies have lower PL since the rhabdomeres are reduced (7). We expected that *ora* would have lower PL since this mutant lacks the R1-6 rhabdomeres. Surprisingly, there were actually higher PL ratios in this mutant, suggesting that there may be a compensatory increase in PL in parts of the head other than the eyes. With the exception of a decrease in PC and PC_r, other membrane PL in *norpA* were not different from those of the wild type. This finding was expected because the *norpA* flies were newly emerged, while rhabdomere diminution is only realized in aged mutants (15). On the other hand, there was a large increase in TG in *norpA* as compared to controls. The cause for this increase is not clear.

The acyl group profiles of individual PL (PS, PI, PE, PC, PC_r) and TG in heads of newly-eclosed wild-type *Drosophila* reared on the regular diet are shown in Table 3. Although subtle differences could be found among the PL, the overall profiles were similar with high proportions of 18:1, 18:2 and, in some cases, 18:3, the latter two being very low in the regular food (Table 1). On the other hand, the acyl group profile of TG is in many ways different from that of PL. Similar to the TG acyl profiles of mammalian systems, the hallmark of the TG profile of *Drosophila* heads is the near absence of 18:0. On the other hand, the TG in *Drosophila* heads showed a high proportion of 14:0, a fatty acid which was below detection level in the food. An unknown lipid localized to the right of PC on the HPTLC plates (PC_r) has a greater proportion of the short chain saturated fatty acids (14:0 and 16:0) than PC. These data suggest that this newly resolved PL spot is part of the PC species. One conspicuous finding is that there are no fatty acid chains longer than 18 carbons in the PL and TG of *Drosophila* heads, confirming and extending the observations of Yoshioka *et al.* (14).

Since PE is the predominant PL present in the *Drosophila* head, we compared the acyl group profile of PE for different types of *Drosophila* (newly-eclosed wild-type, newly-eclosed *norpA*, newly-eclosed *eya*, aged wild-type, aged *ora*, and carotenoid replete and deprived wild-type, with only the latter two being reared on Sang's medium). As shown in Table 4, all types fed the regular diet showed similar fatty acid profiles. That is, aging and mutant condition did not change the profile of acyl groups in PE. Results from PI, not presented, are much like those for PE. The most striking diffrences result from comparing the flies fed the regular diet *vs.* the Sang's medium. Flies reared on Sang's medium showed significantly lower

TABLE 2

Ratio of Lipid in Experimental *vs.* Control Heads

Lipid[a]	*eya*[b]	*ora*[c]	*norpA*[d]	Vit. A deprived[e]
PE	0.46	1.09	1.08	0.76
PC	0.65	1.38	0.69	0.65
PC_r	—	—	0.62	—
PI	0.56	2.00	0.96	0.65
PS	0.58	1.30	1.08	0.52
TG	—	—	2.54	—

[a] PE, phosphatidylethanolamine; PC, phosphatidylcholine; PC_r, lipid fraction migrating to the right of PC; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triglyceride.

[b] *e*yes *a*bsent, control is wild-type, both newly-eclosed, regular diet.

[c] *o*uter *r*habdomeres *a*bsent, control is wild-type, both aged, regular diet.

[d] *no* receptor *p*otential, control is wild-type, both newly-eclosed, regular diet.

[e] Control is vitamin A replete, both wild type, Sang's diet.

TABLE 3

Percentages of Fatty Acids in the Lipids of Heads of Newly-Eclosed Wild-Type Flies Reared on Regular Diet

Fatty acid	PS[a]	PI[b]	PE[c]	PC[d]	PC_r[e]	TG[f]
			mole %			
14:0	3.0[g] (0.3)	—	0.6 (0.1)	2.0 (0.01)	6.8 (0.9)	23.0 (1.0)
16:0	9.4 (0.1)	17.6[h]	18.1 (0.6)	24.8 (1.0)	39.0 (3.1)	21.3 (1.0)
16:1	3.0 (0.2)	6.6	7.3 (0.3)	11.5 (0.6)	—	26.7 (1.0)
18:0	11.9 (0.5)	10.8	11.0 (0.2)	8.0 (1.7)	14.6 (1.2)	0.8 (0.3)
18:1	32.5 (0.2)	25.3	24.2 (0.6)	24.4 (1.8)	18.3 (2.9)	22.6 (1.0)
18:2	24.5 (0.1)	29.6	28.8 (0.7)	26.5 (1.8)	17.1 (4.4)	3.2 (0.5)
18:3	15.8 (0.2)	10.1	10.2 (0.5)	3.0 (0.2)	—	0.3 (0.1)

[a] Phosphatidylserine, averaged from three experiments.

[b] Phosphatidylinositol, averaged from four experiments.

[c] Phosphatidylethanolamine, averaged from four experiments.

[d] Phosphatidylcholine, averaged from three experiments.

[e] Lipid fraction migrating to the right of phosphatidylcholine, one experiment.

[f] Triglyceride, averaged from 2 experiments.

[g] Values averaged from 3 samples (± standard deviation).

[h] Values for PI averaged from 2 samples, no error listed.

TABLE 4

Percentages of Fatty Acids in Phosphatidylethanolamine in Heads of All Fly Types Studied

Fatty acid	Young wild-type[a]	Young *norpA*[b]	Young *eya*[c]	Aged wild-type[a]	Aged *ora*[d]	Vitamin A replete[e]	Vitamin A deprived[e]
				mole %			
16:0	18.1[f] (0.6)	19.4[g]	16.8 (0.5)	17.2 (0.4)	15.4 (0.8)	16.9 (0.2)	16.3 (0.8)
16:1	7.3 (0.3)	6.6	12.0 (0.2)	11.4 (0.6)	13.0 (0.5)	30.0 (0.4)	27.9 (1.7)
18:0	11.0 (0.2)	16.3	8.9 (0.5)	8.9 (0.5)	10.4 (0.3)	5.3 (0.02)	6.4 (0.4)
18:1	24.2 (0.6)	26.5	28.3 (0.9)	25.0 (1.0)	25.0 (2.1)	41.4 (1.0)	41.4 (2.0)
18:2	28.8 (0.7)	24.2	21.9 (0.4)	27.0 (0.3)	25.1 (1.1)	4.9 (0.4)	6.1 (0.5)
18:3	10.2 (0.5)	6.7	12.2 (0.8)	10.5 (0.6)	11.2 (0.9)	1.5 (0.04)	2.0 (0.2)

[a]Regular diet; "young" = newly-eclosed; "aged" = one week post-eclosion.
[b]*no* receptor *p*otential, regular diet, newly-eclosed.
[c]*e*yes *a*bsent, regular diet, newly-eclosed.
[d]*o*uter *r*habdomeres *a*bsent, regular diet, one week post-eclosion.
[e]Sang's diet, newly-eclosed.
[f]Values averaged from three samples (± standard deviation).
[g]Values for young *norpA* averaged from two samples, no error listed.

TABLE 5

Percentages of Fatty Acids in Triglyceride in Heads of All Fly Types Studied

Fatty acid	Young wild-type[a]	Young *norpA*[b]	Young *eya*[c]	Aged wild-type[a]	Aged *ora*[d]	Vitamin A replete[e]	Vitamin A deprived[e]
				mole %			
14:0	23.0[f] (1.0)	23.2 (3.6)	18.4 (1.1)	15.9[g]	26.8	21.1 (3.1)	16.4 (1.2)
14:1	2.1 (0.1)	2.4 (0.3)	1.9 (0.1)	1.6	2.5	—	—
16:0	21.3 (1.0)	22.5 (1.2)	12.0 (0.4)	12.7	10.5	12.9 (0.6)	9.4 (0.4)
16:1	26.7 (1.0)	23.5 (0.5)	15.4 (0.4)	16.7	13.2	11.6 (0.5)	7.7 (0.5)
18:0	0.8 (0.3)	0.8 (0.1)	—	—	—	—	—
18:1	22.6 (1.0)	24.0 (2.0)	49.4 (1.3)	50.1	44.6	51.9 (4.1)	63.1 (0.8)
18:2	3.2 (0.5)	3.3 (0.4)	2.9 (0.1)	3.2	2.5	2.5 (0.1)	3.3 (0.2)
18:3	0.3 (0.5)	0.5 (0.1)	—	—	—	—	—

[a–f]As in Table 4.
[g]Values for aged wild-type and aged *ora* averaged from two samples, no error listed.

proportions of 18:2 and 18:3 and higher proportions of 16:1 and 18:1 than those raised on the regular food; recall that the Sang's medium had higher 18:1 (Table 1).

The acyl group profiles for TG for the same fly types are presented in Table 5. In contrast with the acyl profile for PE, TG fatty acids tend to show large differences with age and mutant condition. The Sang's medium flies showed strikingly high 18:1. Interestingly, 18:1 was also very high in *eya* flies, which might imply that it is correspondingly low in the visual system which *eya* flies lack. Finally, the aged flies have a higher proportion of 18:1 and lower proportions of 16:0 and 16:1 in the TG (Table 5). Recall that we had originally decided to study aged wild-type flies as controls in the experiment with aged *ora* since *ora* loses its rhabdomeres as a function of age (12).

DISCUSSION

One of our primary findings is that the fatty acid content of *Drosophila* heads can readily be altered by the diet. Large differences in fatty acid composition for PL and TG were observed by comparing flies reared on the Sang's diet (for vitamin A manipulations) *vs.* the regular diet. Our data are in line with the statement by Turunen (16) that "the tissue fatty acid composition often is quite sensitive to dietary changes." A comparison of the fatty acids from the food *vs.* those from *Drosophila* suggests that the flies cannot elongate the fatty acids to make ≥20 carbon compounds but they can desaturate the fatty acids, *e.g.*, from 16:0 to 16:1.

Our results are in general agreement with those reported by Yoshioka *et al.* (14) who determined the fatty acid composition of PI and PC in *Drosophila*. Both studies concur in that there are no long chain polyunsaturated fatty acids in the tissue PL. The small discrepancies can well be attributed to differences in the diet since the diet used by Yoshioka *et al.* had less 16:0, did have 16:1 and had higher 18:0, 18:1 and 18:2. They obtained higher values of C_{16} fatty acids and lower values of C_{18} fatty acids in PI and the reverse for PC compared to ours. Their analysis showed the presence of small amounts of C_{15} and C_{17} fatty acids, but we did not find them in our study. On the other hand, we observed small amounts of 18:3 while they did not.

It would be interesting to determine whether dietary supplementation with long chain fatty acids may have any effects on the fatty acids of the visual system. In a preliminary study, R. Sapp and W. S. Stark (unpublished) found that supplementation of the regular food with menhaden oil (containing a high proportion of polyunsaturated fatty acids) had no obvious effect on visual receptor ultrastructure. On the other hand, D.-M. Chen and W. S. Stark (unpublished) showed a small increase in electroretinographic sensitivity of R1-6 from this supplementation, especially in the near UV region of the spectrum. These data suggest that the fly model will prove useful to elucidate the relationship between dietary fatty acids and visual function.

Vertebrate visual receptors contain high proportions of the long chain polyunsaturated fatty acids, especially 22:6 and 20:4 (17). Such high levels of long chain fatty acids in vertebrate visual receptors are of substantial interest (18). Although the function of 22:6 (DHA) in vertebrate photoreceptors is not known (19,20), the conventional wisdom is that unsaturated fatty acids contribute to membrane fluidity which could facilitate interactions of the molecules of the signal transduction cascade in the planar disk membranes of the rod outer segment. A defect in metabolism or transport of DHA in poodles afflicted with progressive rod-cone degeneration suggests that DHA is essential for visual receptor maintenance in vertebrates (21). *Drosophila* may prove helpful in determining the function of DHA since DHA is absent in the lipids of flies whose vision is intact. In contrast to the fly, the cabbage butterfly *Pieris brassicae* incorporates 20:5n-3 (eicosapentaenoic acid) into a variety of lipids including PI (22). Insects provide a wide natural diversity in diet potentially providing a wealth of comparative biochemistry. For instance, some insects are "phytophagous," meaning that they feed on plants, and, in many cases, the insect-host relationship is very specific, while others feed on meat or blood, which are rich sources of long chain fatty acids.

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O-Alkyl Diol *O*-, *S*- and *Se*-Phosphoroamidates of DL-α-Tocopherol and Their Dimethylaminoalkyl Derivatives as Diester and Triester Models of Phospholipids

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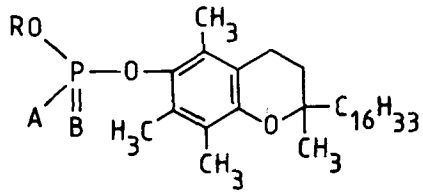
Hexamethyltriamide of phosphorous acid, activated by addition of iodine at an optimal molar ratio of 1.05:0.05, was used as a phosphorylating reagent to synthesize 1-hexadecyloxyethyl-2-*O*-, 1-hexadecyloxypropyl-3-*O*-, and 1-hexadecyloxybutyl-4-*O*-(DL-α-tocopheryl-6-*O*)-(*N*,*N*-dimethylamido)selenophosphate, -thiophosphate and -phosphate derivatives, and some of their 2-dimethyl-aminoethyl-1-*O*-, and 3-dimethylaminopropyl-1-*O*-triester analogues in a "one-pot procedure" in overall yields of 69–87%. Activation of the reaction with an equimolar mixture of imidazole and carbon disulfide at the triester formation step permits selective phosphorylation at room temperature. The compounds synthesized represent new diester and triester models containing alkyl ether diolphospholipid structures. *Lipids 28,* 351–354 (1993).



1ad0; 1bdS; 1cdSe; 1adeS; 1cdgSe

FIG. 1. For 1adO: R = 1-Hexadecyloxyethyl-; A = $N(CH_3)_2$; B = O; 1bdS: R = 1-hexadecyloxypropyl-; A = $N(CH_3)_2$; B = S; 1cdSe: R = 1-hexadecyloxybutyl-; A = $N(CH_3)_2$; B = Se; 1adeS: R = 1-hexadecyloxyethyl-; A = 2-dimethylaminoethyl-*O*-; B = S; 1cdgSe: R = 1-hexadecyloxybutyl-; A = 3-dimethylamino-1-propyl-*O*-; B = Se.

O-Alkyl diolphospholipids and polar lipids containing phosphate triester linkages are important mediators of a variety of biological activities (1–4). Diester types of naturally occurring and of model alkyl ether diolphospholipids have been obtained synthetically to elucidate their chemical and pharmacological properties (4–7). Triester derivatives, or thio- and selenophosphate analogues of alkyl ether diolphospholipids have not been reported to date. Thiophosphate model analogues of other naturally occurring phospholipids, however, are now well recognized as advantageous probes in biochemical and membrane studies (8,9).

Further developments in diolphospholipid research will be stimulated by the design of novel chemical structures that include (in addition to a diol moiety) a membrane active lipid (steroid, tocopherol, etc.) joined *via* a phosphate bridge. This new and challenging approach is expected to contribute to progress in biochemistry, pharmacology and membrane science (10,11).

In the present paper we describe the synthesis of alkyl esters of diol *O*-, *S*- and *Se*-phosphoroamidates of DL-α-tocopherol and some of their dimethylaminoalkyl triester analogues (Fig. 1). These compounds represent model types of ether diolphospholipids that have not been reported previously.

MATERIALS AND METHODS

The tris(*N*,*N*-dimethyl)amide of phosphorous acid (1) was prepared as described by Burg and Slota (12). 1-Hexadecyloxyethan-2-ol (a), 1-hexadecyloxy-propan-3-ol (b) and 1-hexadecyloxybutan-4-ol (c) were synthesized as described by Tsushima *et al.* (4). DL-α-Tocopherol (d), 2-dimethylaminoethanol (e), 1-dimethylamino-2-propanol (f) and 3-dimethylamino-1-propanol (g) (Fluka, Buchs, Switzerland; and Merck, Darmstadt, Germany) had a purity of more than 98%. All other reagents were purchased from Janssen (Stockholm, Sweden) and were better than 98% pure. Benzene (Merck) was dried over sodium and freshly distilled prior to use. Reaction conditions were kept strictly anhydrous.

Analytical thin-layer chromatography (TLC) on precoated aluminum sheets of Silica Gel 60 F_{254} (Merck) was routinely used for monitoring reactions. High-performance liquid chromatography (HPLC) was done (Gilson 305 System, equipped with a Gilson 131 refractive index detector; Medical Electronics, Middleton, WI) using a Polygosil 60-7 silica gel column (Scandinaviska Genetik AB, Sweden; 250 × 10 mm). Chloroform (System A), *n*-hexane/diethyl ether (20:80, vol/vol; System B), *n*-heptane/ethyl acetate (80:20, vol/vol; System C) and chloroform/methanol (90:10, vol/vol; System D) were used as mobile phases.

^{13}C Nuclear magnetic resonance (NMR) spectra were recorded on a Varian (Palo Alto, CA) XL-300 spectrometer at 75.43 MHz. ^{13}C Chemical shifts are reported in ppm relative to tetramethylsilane (TMS). ^{31}P NMR spectra were recorded on the same instrument at 121.42 MHz. ^{31}P Chemical shifts are reported in ppm relative to 85% phosphoric acid (external) where a positive sign is downfield from the standard. All ^{13}C and ^{31}P NMR data given refer to proton decoupled spectra. Infrared (IR) spectra were recorded on a Perkin-Elmer (Beaconsfield, England) FT-IR 1750 spectrometer. Peak positions are reported in cm^{-1}. Satisfactory microanalyses were obtained for **1adO, 1bdS, 1cdSe, 1adeS,** and **1cdgSe:** C, ±0.21; H, ±0.11; N, ±0.08; P, ±0.10; S, ±0.10.

1-Hexadecyloxyethyl-2-O-(DL-α-tocopheryl-6-O)-(N,N-dimethylamido)phosphate, ***1adO.*** *Representative proce-*

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Abbreviations: HPLC, high-performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

dure. A mixture of iodine (0.025 g, 0.1 mmol) and the *tris* (*N,N*-dimethyl)amide of phosphorous acid (**1**; 0.343 g, 2.1 mmol) in benzene (50 mL) was heated at 70°C in a stream of argon for about 15 min until the precipitate dissolved. 1-Hexadecyloxyethan-2-ol (**a**; 0.576 g, 2.0 mmol) was added and the mixture was kept under these conditions for 5 min. Then DL-α-tocopherol (**d**; 0.861 g, 2.0 mmol) was added and the reaction mixture was heated at 70°C for 2 h. The resultant diester phosphite **1ad** was transformed to the phosphate **1adO** by reaction with benzoyl peroxide (with 20% water) (0.636 g, 2.1 mmol) at room temperature (20–25°C) for 10 min. The solvent was removed under vacuum, and the compounds was isolated by HPLC (System C) in pure form. Yield of **1adO**: 1.40 g (87%); $n_D^{40} = 1.4787$; R_f (System A), 0.11; Anal. Calcd. for $C_{49}H_{92}NO_5P$ (806.4): C, 72.98; H, 11.52; N, 1.74; P, 3.84. Found: C, 72.77; H, 11.60; N, 1.80; P, 3.90. ^{13}C NMR ($CDCl_3$) 1-hexadecyloxyethyl-2-*O*-fragment: δ 11.9 ppm (C-16); 23.9 (C-15); 26.1 (C-2); 29.4–29.7 (*m*, C-5 to C-13); 31.9 (C-14); 65.4 (*d*, $CH_2\underline{C}H_2OP$, $J = 6$ Hz); 69.7 (*d*, $\underline{C}H_2CH_2OP$, $J = 8$ Hz); 71.4 (C-1); DL-α-tocopheryl-6-*O*-fragment: 13.0 (5-CH_3); 13.9 (7-CH_3); 14.1 (8-CH_3); 20.8 (C-4); 21.0 (C-3); 23.8 (2-CH_3); 74.8 (C-2); 140.9 (*d*, C-6, $J = 9$ Hz); 148.4 (C-9) (nucleus); 19.6 (C-13, 12-CH_3); 22.6, 22.7 (4-CH_3, 8-CH_3) (chain); 36.9 (*m*, CH_3N). ^{31}P NMR ($CDCL_3$) δ 7.9 ppm (*s*). IR (KBr, film) ν 1245, 836 (PO-C, P-OC_{aryl}); 1191 (P = O); 1050, 791 (PO-C, P-OC); 735 cm^{-1} (P-N).

1-Hexadecyloxypropyl-3-O-(DL-α-tocopheryl-6-O)-(N,N-dimethylamido)thiophosphate ***1bdS.*** The intermediate **1bd** was prepared using 1-hexadecyloxypropan-3-ol (**b**, 0.601 g, 2.0 mmol) and DL-α-tocopherol (**d**, 0.861 g, 2.0 mmol) as described for **1adO.** Transformation to the thiophosphate **1bdS** was accomplished by reaction with sulfur (0.067 g, 2.1 mmol) at 70°C for 3 min. The derivative was isolated by HPLC (System C) as described for **1adO.** Yield of **1bdS**: 1.41 g (84%); $n_D^{40} = 1.4909$; R_f (System A), 0.66; Anal. Calcd. for $C_{50}H_{94}NO_4PS$ (836.5): C, 71.78; H, 11.35; N, 1.67; P, 3.71; S, 3.83. Found: C, 71.85; H, 11.24; N, 1.67; P, 3.66; S, 3.93. ^{13}C NMR ($CDCl_3$) 1-hexadecyloxypropyl-3-*O*- fragment: δ 12.0 ppm (C-16); 23.9 (C-15); 26.3 (C-2); 29.5–29.8 (*m*, C-5 to C-13); 30.6 (*d*, $CH_2\underline{C}H_2CH_2OP$, $J = 9$ Hz); 32.0 (C-14); 63.7 (*d*, $CH_2CH_2\underline{C}H_2OP$, $J = 6$ Hz); 66.9 ($\underline{C}H_2CH_2CH_2OP$); 71.2 (C-1); DL-α-tocopheryl-6-*O*- fragment: 13.4 (5-CH_3); 14.2 (7-CH_3); 14.3 (8-CH_3); 20.8 (C-4); 21.1 (C-3); 23.8 (2-CH_3); 74.7 (C-2); 142.0 (*d*, C-6, $J = 9$ Hz); 148.4 (C-9) (nucleus); 19.7 (C-13, 12-CH_3); 22.7 (4-CH_3, 8-CH_3) (chain); 37.4 (*m*, CH_3N). ^{31}P NMR ($CDCl_3$) δ 74.8 ppm (*s*). IR (KBr, film) ν 1246, 834 (PO-C, P-OC_{aryl}); 1050, 792 (PO-C, P-OC); 747 (P-N); 719 cm^{-1}(P = S).

1-Hexadecyloxybutyl-4-O-(DL-α-tocopheryl-6-O)-(N,N-dimethylamido)selenophosphate ***1cdSe.*** The phosphite **1cd** was prepared using 1-hexadecyloxybutan-4-ol (**c**, 0.629 g, 2.0 mmol) and DL-α-tocopherol (**d**, 0.861 g, 2.0 mmol) following the procedure described for **1adO.** Then selenium powder (0.166 g, 2.1 mmol) was added and the mixture was stirred at 70°C for 4 h. The selenophosphate **1cdSe** was isolated as described for **1adO.** Yield of **1cdSe**: 1.43 g (80%); $n_D^{40} = 1.4942$; R_f (System A), 0.67; Anal. Calcd. for $C_{51}H_{96}NO_4PSe$ (897.4): C, 68.25; H, 10.80; N, 1.56; P, 3.45. Found: C, 68.22; H, 10.83; N, 1.53; P, 3.35. ^{13}C NMR ($CDCl_3$) 1-hexadecyloxybutyl-4-*O*- fragment: δ 12.0 ppm (C-16); 23.8 (C-15); 26.2 ($CH_2\underline{C}H_2CH_2CH_2OP$); 26.3 (C-2); 26.9 (*d*, $CH_2CH_2\underline{C}H_2CH_2OP$, $J = 9$ Hz); 29.5–29.8 (*m*, C-5 to C-13); 32.0 (C-14); 67.0 (*d*, $CH_2\underline{C}H_2OP$, $J = 5$ Hz); 70.1 ($\underline{C}H_2CH_2CH_2CH_2OP$); 71.0 (C-1); DL-α-tocopheryl-6-*O*-fragment: 13.6 (5-CH_3); 14.2 (7-CH_3); 14.4 (8-CH_3); 20.8 (C-4); 21.0 (C-3); 23.8 (2-CH_3); 74.8 (C-2); 142.3 (*d*, C-6, $J = 9$ Hz); 148.5 (C-9) (nucleus); 19.7 (*m*, C-13, 12-CH_3); 22.7 (*m*, 4-CH_3, 8-CH_3) (chain); 37.4 (*m*, CH_3N). ^{31}P NMR ($CDCl_3$) δ 79.7 ppm (*t*, $J_{P-Se} = 457$ Hz). IR (KBr, film) ν 1245, 832 (PO-C, P-OC_{aryl}); 1041, 784 (PO-C, P-OC); 752 (P-N); 720 cm^{-1} (P = Se).

1-Hexadecyloxyethyl-2-O-(DL-α-tocopheryl-6-O)-(2-dimethylaminoethyl-1-O-)thiophosphate ***1adeS.*** *Representative procedure.* The diester **1ad** was prepared using 1-hexadecyloxyethan-2-ol (**a**, 0.576 g, 2.0 mmol) and DL-α-tocopherol (**d**, 0.861 g, 2.0 mmol) as described for **1adO.** The solution was cooled to room temperature (20–25°C) and added to a mixture of 2-dimethylaminoethanol (**e**, 0.178 g, 2.0 mmol), imidazole (0.136 g, 2.0 mmol) and carbon disulfide (0.152 g, 2.0 mmol) in benzene (50 mL). After 7 h at 20–25°C, the resultant triester phosphite **1ade** was transformed to the thiophosphate **1adeS** by reacting with sulfur (0.067 g, 2.1 mmol) for 30 min at the temperature indicated. The solvent was removed under vacuum, and the derivative was isolated by HPLC (System D) in pure form. Yield of **1adeS**: 1.23 g (71%); $n_D^{40} = 1.4859$; R_f (System B), 0.23; Anal. Calcd. for $C_{51}H_{96}NO_5PS$ (866.5): C, 70.68; H, 11.19; N, 1.62; P, 3.58; S, 3.70. Found: C, 70.62; H, 11.22; N, 1.65; P, 3.48; S, 3.75. ^{13}C NMR ($CDCl_3$) 1-hexadecyloxyethyl-2-*O*- fragment: δ 11.9 ppm (C-16); 23.9 (C-15); 26.1 (C-2); 29.4–29.7 (*m*, C-5 to C-13); 31.9 (C-14); 66.1 (*d*, $CH_2\underline{C}H_2OP$, $J = 6$ Hz); 69.4 (*d*, $\underline{C}H_2CH_2OP$, $J = 8$ Hz); 71.5 (C-1); DL-α-tocopheryl-6-*O*- fragment: 13.5 (5-CH_3); 14.1 (7-CH_3); 14.4 (8-CH_3); 20.8 (C-4); 21.0 (C-3); 23.8 (2-CH_3); 74.9 (C-2); 141.6 (*d*, C-6, $J = 9$ Hz); 148.7 (C-9) (nucleus); 19.7 (*m*, C-13, 12-CH_3); 22.7 (*m*, 4-CH_3, 8-CH_3) (chain); 2-dimethylaminoethyl-1-*O*- fragment: 45.5 (CH_3N); 58.5 (CH_2N); 67.6 (*d*, CH_2OP, $J = 6$ Hz). ^{31}P NMR ($CDCl_3$) δ 65.3 ppm (*s*). IR(KBr, film) ν 1246, 837 (PO-C, P-OC_{aryl}); 1040; 818 (PO-C, P-OC); 722 cm^{-1} (P = S).

1-Hexadecyloxybutyl-4-O-(DL-α-tocopheryl-6-O)-(3-dimethylaminopropyl-1-O-)selenophosphate ***1cdgSe.*** The phosphite **1cdg** was prepared using 1-hexadecyloxybutan-4-ol (**c**, 0.629 g, 2.0 mmol), DL-α-tocopherol (**d**, 0.861 g, 2.0 mmol) and 3-dimethylamino-1-propanol (**g**, 0.206 g, 2.0 mmol) following the procedure described for **1adeS.** Transformation to the selenophosphate **1cdgSe** was accomplished by reaction with selenium (0.166 g, 2.1 mmol) at 70°C for 5 h. The crude derivative was purified by HPLC (System D). Yield of **1cdgSe**: 1.32 g (69%); $n_D^{40} = 1.4873$; R_f (System B), 0.28; Anal. Calcd. for $C_{54}H_{102}NO_5PSe$ (955.5): C, 67.87; H, 10.78; N, 1.47; P, 3.24. Found: C, 67.83; H, 10.75; N, 1.48; P, 3.24. ^{13}C NMR ($CDCl_3$) (Fig. 2) 1-hexadecyloxybutyl-4-*O*- fragment: δ 11.9 ppm (C-16); 23.8 (C-15); 25.9 ($CH_2\underline{C}H_2CH_2CH_2OP$); 26.3 (C-2); 27.0 (*d*, $CH_2CH_2\underline{C}H_2CH_2OP$, $J = 8$ Hz); 29.4–29.7 (*m*, C-5 to C-13); 31.9 (C-14); 67.4 (*d*, $CH_2\underline{C}H_2OP$, $J = 5$ Hz); 70.0 ($\underline{C}H_2CH_2CH_2CH_2OP$); 71.0 (c-1); DL-α-tocopheryl-6-*O*- fragment: 13.8 (5-CH_3); 14.1 (7-CH_3); 14.6 (8-CH_3); 20.8 (C-4); 21.0 (C-3); 23.8 (2-CH_3); 74.8 (C-2); 141.9 (*d*, C-6, $J = 9$ Hz); 148.7 (C-9) (nucleus); 19.7 (*m*, C-13, 12-CH_3); 22.7 (*m*, 4-CH_3, 8-CH_3) (chain); 3-dimethylaminopropyl-1-*O*- fragment: 28.3 (*d*, $\underline{C}H_2CH_2OP$, $J = 8$ Hz); 45.4 (CH_3N); 55.8 (CH_2N); 69.0 (*d*, $\underline{C}H_2OP$, $J = 5$ Hz). ^{31}P NMR ($CDCl_3$) δ 68.8 ppm (*t*,

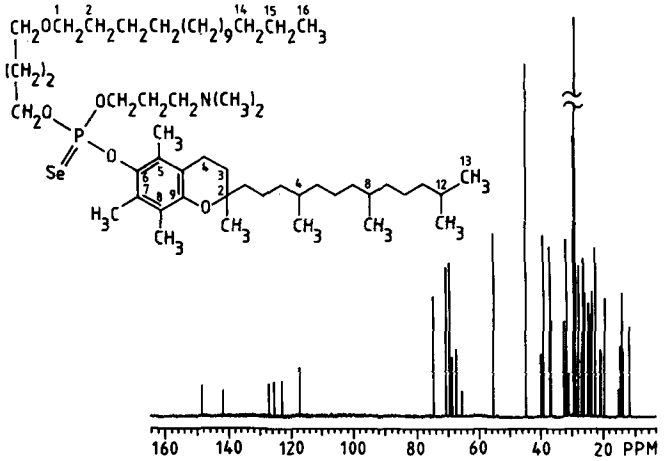

FIG. 2. Proton-decoupled 75.43 MHz ^{13}C nuclear magnetic resonance spectrum of 1-hexadecyloxybutyl-4-*O*-(DL-α-tocopherol-6-*O*)-(3-dimethylaminopropyl-1-*O*)selenophosphate, 1cdgSe.

J_{P-Se} = 453 Hz). IR (KBr, film) ν 1245, 834 (PO-C, P-OC_{aryl}); 1039, 789 (PO-C, P-OC); 735 cm^{-1} (P = Se).

RESULTS AND DISCUSSION

As it has been shown, the acyclic triamides of phosphorous acid offer a number of advantages over conventional phosphorylating reagents (10,11). Moreover, these phosphamides, after appropriate activation, can also be subjected to stoichiometric alcoholysis under mild conditions to give mono-, di- and triester phosphites in high yields (13–15).

The *tris*(*N,N*-dimethyl)amide of phosphorous acid with iodine at the optimum molar ratio of 1.05:0.05 was used as base reagent, **1**. 1-Hexadecyloxyethan-2-ol **a**, 1-hexadecyloxypropan-3-ol **b**, 1-hexadecyloxybutan-4-ol **c**, DL-α-tocopherol **d**, 2-dimethylaminoethanol **e**, 1-dimethylamino-2-propanol **f** and 3-dimethylamino-1-propanol **g** were selected as substrates for phosphorylation. The synthesis was performed according to the method we recently proposed (13,14).

The diesters **1ad, 1bd, 1cd** were obtained in a "one-pot" procedure (Fig. 3) by consecutive treatment of the reagent **1** with equivalent quantities of the ether diols **a, b, c** and DL-α-tocopherol **d** at 70°C in high yields. This was proven by transformation of the phosphites to the corresponding phosphate **1adO**, thiophosphate **1bdS**, and selenophosphate **1cdSe** derivatives. The reverse phosphorylation sequence (DL-α-tocopherol first, and then an ether diol) led to transesterification, probably for both steric and chemical reasons. The reaction sequence established earlier for the synthesis of diol S- and O-phosphoroamidates of some sterols supports this explanation. The molecular masses of the diols chosen had no effect on the rates of phosphorylation.

The high selectivity of diester formation permits the direct synthesis of unsymmetrical triesters. Thus, an equimolar mixture of the third substrate, *e.g.*, imidazole and carbon disulfide, was treated in stoichiometric proportions with the crude diester intermediates **1ad, 1cd** at room temperature for 7 h (Fig. 4). Treating the reaction

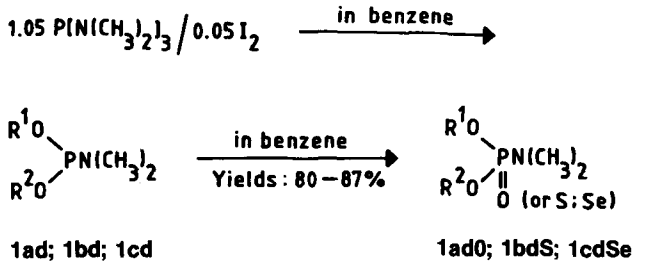

FIG. 3. For the synthesis of 1ad, 1bd, 1cd: 1.) R^1OH = a, b, or c (70°C/5 min); 2.) R^2OH = d (70°C/2 h); for the synthesis of 1adO: 3.) $(C_6H_5CO)_2O_2$ (20–25°C/10 min); or 1bdS: S_8 (70°C/3 min); or 1cdSe: Se (70°C/4 h). For a, 1ad, 1adO: R^1 = 1-hexadecyloxyethyl-; b, 1bd, 1bdS: R^1 = 1-hexadecyloxypropyl-; c, 1cd, 1cdSe: R^1 = 1-hexadecyloxybutyl-; d, 1ad, 1bd, 1cd, 1adO, 1bdS, 1cdSe: R^2 = DL-α-tocopheryl-.

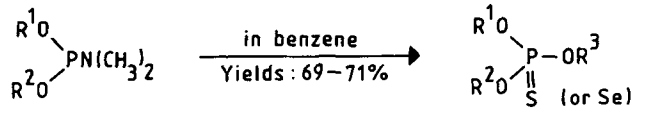

FIG. 4. For the synthesis of 1adeS, 1cdgSe: 1.) R^3OH = e, g/Im/CS_2 (20–25°C/7 h); 2.) S_8 (20–25°C/30 min); or Se (70°C/5 h). For 1ad, 1adeS: R^1 = 1-hexadecyloxyethyl-; R^2 = DL-α-tocopheryl-; 1cd, 1cdgSe: R^1 = 1-hexadecyloxybutyl-; R^2 = DL-α-tocopheryl-; e, 1adeS: R^3 = 2-dimethylaminoethyl-; g, 1cdgSe: R^3 = 3-dimethylamino-1-propyl-; Im = imidazole.

product then with sulfur, or selenium, generated the triester thiophosphate **1adeS**, or the selenophosphate **1cdgSe** (Fig. 2), in good overall yields.

Our attempts to obtain the 1-hexadecyloxypropyl-3-*O*-(1-dimethylaminopropyl-2-*O*) triester derivative of DL-α-tocopherol failed. Despite extended reaction times (up to one week), at the last synthetic step the third substrate **f** was not coupled with the activated diester intermediate **1bd**. The 1-dimethylamino-2-propanol **f** was not reactive either toward the other diester phosphites **1ad, 1cd**. This is probably due to the steric hindrance at the methyl substituted secondary hydroxyl function of **f**.

As a rule, reaction with selenium required relatively longer reaction times than oxidation or reaction with sulfur. No attempts were made to optimize this process.

Conclusions on the structures of the compounds synthesized were drawn on the basis of spectral and microanalytical data. ^{13}C NMR indicated that all characteristic signals present in the spectra of the starting substrates were also seen in the spectra of the corresponding target *O-*, *S-* and *Se*-phosphate derivatives. No chemical alterations in the alkyl ether diol (**a, b, c**), tocopheryl (**d**) and dimethylaminoalkyl (**e, g**) moieties were detected after oxidation, sulfurization or selenization. The ^{31}P NMR spectral analysis provided compelling evidence that the latter processes concerned the P^{III} atom, exclusively, under the specific experimental conditions selected. These results are in agreement with those reported in the literature for analogous systems (7,11,15).

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METHOD

Use of Acetyl Chloride/Methanol for Assumed Selective Methylation of Plasma Nonesterified Fatty Acids Results in Significant Methylation of Esterified Fatty Acids

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The albumin-bound nonesterified fatty acid pool in plasma, which represents a very small percentage of total plasma fatty acids, has previously been quantitated by a variety of methods. In the present study we determined that the nonesterified fatty acid concentrations in the plasma, quantitated by a popular method using acetyl chloride and methanol which is reported to be specific for methylation of nonesterified fatty acids in the presence of esterified fatty acids (*i.e.*, without prior isolation of the plasma nonesterified fatty acids), were significantly overestimated due to cleavage and methylation of esterified fatty acids. Quantitation of the contaminating fatty acid from the esterified pool demonstrated that the amount of fatty acid cleaved from the esterified pool was enough to exceed the entire mass of nonesterified fatty acids. As an established method for comparison, we isolated nonesterified fatty acids from the plasma by thin-layer chromatography prior to methylation, using a number of simple precautions to limit oxidation. By performing all thin-layer chromatography steps in an atmosphere of nitrogen and by including fatty acid standards in the plasma with 0, 1, 2 or 4 double bonds, we were able to accurately and reproducibly determine the concentration of nonesterified fatty acids in the plasma, including arachidonate. We demonstrated that no oxidation occurred in the thin-layer chromatographic isolation of nonesterified fatty acids and that the coefficients of variation for repeat measurements of the same sample were <11% using our reference method. Our data indicate that the use of acetyl chloride and methanol for assumed selective methylation of plasma nonesterified fatty acids results in significant methylation of esterified fatty acids. *Lipids 28*, 355–360 (1993).

Nonesterified fatty acids (NEFA) bound to albumin comprise a very small percentage of the total plasma fatty acids. Although the size of this pool changes with diet, the percentage of the nonesterified fatty acids relative to total plasma fatty acids rarely exceeds 5% (1). The total nonesterified fatty acid pool has been quantitated by a variety of methods including colorimetric (2,3) and enzymatic (4–8) assays.

The quantitation of the small pool of plasma nonesterified fatty acids without contamination by the much larger pool of esterified fatty acids has led many investigators to isolate the plasma nonesterified fatty acids before quantitation. However, the isolation of nonesterified fatty acids by methods such as thin-layer chromatography (TLC) is time consuming and without special precautions being taken to minimize oxidation can result in significant loss of the polyunsaturated fatty acids, particularly arachidonate. To address this problem, a number of simple methods for measuring nonesterified fatty acids have been developed which do not involve prior nonesterified fatty acid isolation. One of these popular methods involving the use of acetyl chloride and methanol was developed by Lepage and Roy (7). In the present study we attempted to use that method to quantitate nonesterified arachidonate in the plasma. Although the method was very efficient in methylating nonesterified fatty acids and was easy to perform, we found that the plasma concentrations of nonesterified arachidonate, as well as the other fatty acids, were significantly overestimated using this approach. A similar observation has recently been made regarding a presumed selective method for nonesterified fatty acid methylation involving diazomethane, which was developed by Pace-Asciak (8). We therefore used TLC to first isolate plasma nonesterified fatty acids prior to methylation and employed simple precautions (i) to limit oxidation of polyunsaturated moieties by spotting and scraping the TLC plate in an atmosphere of nitrogen, and (ii) to account for all oxidation-induced polyunsaturated fatty acid losses by including a variety of polyunsaturated fatty acids as well as an odd-numbered saturated fatty acid as internal standards. Using this as a reference method, we were able to demonstrate that the acetyl chloride/methanol method developed for nonesterified fatty acid methylation also results in significant methylation of esterified fatty acids.

MATERIALS AND METHODS

Materials. Analytical grade solvents were used in all experiments. Acetyl chloride was purchased from Sigma (St. Louis, MO). Potassium carbonate (anhydrous) was obtained from Fisher (Pittsburgh, PA). Fatty acids, fatty acid methyl esters, triolein, triarachidonin, cholesteryl linoleate and cholesteryl heptadecanoate were purchased from Nu-Chek-Prep (Elysian, MN). Dipalmitoyl phosphatidylcholine and diarachidonoyl phosphatidylcholine were obtained from Avanti (Pelham, AL). Prostaglandin E_2 was a product of Caymen (Ann Arbor, MI). Bovine serum albumin was purchased from Sigma. TLC plates (silica gel 60) were products of E. Merck (Darmstadt, Germany).

Analysis of plasma nonesterified fatty acids. Blood samples were drawn from fasting (for at least 8 h) or nonfasting donors into ethylenediaminetetraacetic acid (EDTA) vacutainers and placed on ice until centrifuged at 4°C. Unless otherwise stated, donors were not fasting. All samples were centrifuged to obtain plasma, and the plasma from each sample was within 10 min after centrifugation frozen at −80°C for periods of up to 1 wk. In separate studies (data not shown), storage under these

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Abbreviations: CE, cholesteryl esters; EDTA, ethylenediaminetetraacetic acid; LDL, low density lipoprotein; NEFA, nonesterified fatty acids; PL, phospholipids; TG, triacylglycerol; TLC, thin-layer chromatography.

conditions was found to have no effect on plasma nonesterified fatty acid concentrations. Plasma NEFA were quantitated in some experiments exactly according to the method of Lepage and Roy (see below) (7). In other experiments, NEFA analysis was performed using a modified Lepage and Roy method in which the temperature or the ratio of methanol to acetyl chloride was varied. Alternatively, NEFA were measured following extraction from plasma by a modified Folch method (9) after addition of known amounts of 17:0 (37 μM), 20:1n-9 (30 μM), 20:2n-6 (26.5 μM) and 22:4n-6 (15 μM) as internal standards. The fatty acids used as standards were not detected in the native plasma nonesterified fatty acid pool (data not shown). The inclusion of 22:4 as an internal standard permitted comparison of recoveries of fatty acids with 0, 1 and 2 double bonds with an internal standard fatty acid possessing 4 double bonds. In all studies, the recovery of the four internal standards was very similar. Thus, there is no reason to believe that arachidonate, isolated from the plasma with higher concentrations of other nonesterified fatty acids, should show a lower recovery than less unsaturated nonesterified fatty acids. In experiments involving NEFA isolation by TLC, lipid extracts were dried under nitrogen at room temperature, resuspended in 0.1 mL of chloroform and spotted onto silica gel 60 TLC plates. During spotting, a gentle stream of nitrogen was kept flowing over the lower half of the plate. The plates were developed in hexane/diethyl ether/acetic acid (70:30:1, by vol) which clearly separated plasma NEFA from other lipids, including phospholipids (PL), triacylglycerol (TG), and cholesteryl esters (CE). Nonesterified fatty acids, PL, TG and CE standards were simultaneously run as standards in parallel lanes on the sample plates. For visualization and scraping, TLC plates were first placed in a dry TLC tank which was continuously flushed with nitrogen. After 5–10 min, when the plate was dry, a glass pasteur pipette filled with iodine crystals connected by tygon tubing to a nitrogen source was inserted into the tank for another 2–5 min until all bands became visible. The iodine was allowed to sublime from the plate before the lipid bands were scraped off the plate. The tank was then tilted on its side, the pipette containing iodine crystals removed, and nitrogen was allowed to continue to flow through the tank. NEFA bands were scraped from the plates in an atmosphere of nitrogen, and the scrapings were immediately added to 5 mL of methanol/acetyl chloride. Excess silica in the mixture was found to reduce the efficiency of methylation. Because there were four internal standards in all test samples, differences in methylating efficiency between experiments had no effect on the final result. The tubes were vortexed for 1 min and then incubated for 45 min at room temperature. During incubation the tubes were vortexed twice for 1 min each time. The methylation reaction was stopped by adding 3 mL of 6% K_2CO_3. Fatty acid methyl esters were extracted into 0.2 mL hexane, dried under nitrogen and resuspended in 5 μL of hexane, from which one μL was injected into a Perkin-Elmer (Norwalk, CT) 8500 gas-liquid chromatograph equipped with a flame ionization detector. Individual fatty acid methyl esters were separated on a WCOT capillary column (Supelcowax 10; Supelco, Bellefonte, PA) using temperature programming from 150 to 250°C at a rate of 10°C/min. Peaks were identified by comparison with the retention times of known standards, and peak areas were integrated. Individual fatty acid masses were determined using internal standards possessing the same number of double bonds. However, in all experiments, because there was no loss of polyunsaturated fatty acids due to oxidation, masses determined for individual nonesterified fatty acids were the same when calculated based on any of the four internal standards.

Quantitation of hydrolysis of esterified fatty acids. Known amounts of dipalmitoyl phosphatidylcholine, diarachidonoyl phosphatidylcholine, tripentadecanoin, triarachidonin, cholesteryl heptadecanoate, cholesteryl linoleate and nonesterified 14:0 (43.8 nmol, 292 μM), 18:0 (35.2 nmol, 234 μM) and 22:4n-6 (15 nmol, 100 μM) were added to a test tube and dried to remove all solvents. Then 0.15 mL of distilled water was added to mimic the aqueous serum component, and the sample was processed using the method of Lepage and Roy (7) exactly as described. Samples of the same lipid mixture were analyzed in parallel for total fatty acid composition using the method of Turk *et al.* (10) to saponify and methylate the esterified fatty acids. For this purpose, the lipid extract was first dried under nitrogen and then resuspended in 0.6 mL of methylene chloride. After vortexing, 1.0 mL of 0.5 M KOH was added, and the mixture allowed to sit for 30 min. Then, 1.0 mL of 6 M HCl was added and, after vortexing, the sample was centrifuged at 100 × *g* for 50 min at room temperature to separate the two phases. The bottom organic layer was aspirated, dried under nitrogen and resuspended in a small volume of hexane for injection into the gas chromatograph. Percent hydrolysis of esterified fatty acids observed by the Lepage and Roy (7) method was calculated by dividing the concentration of methylated fatty acid generated by the Lepage and Roy (7) method by the total esterified fatty acid concentration in the sample for each of the individual lipids tested.

Total plasma fatty acid quantitation. Plasma fatty acids were extracted according to the method of Cohen *et al.* (9) and then methylated using the method of Turk *et al.* (10), followed by the esterification method of Lepage and Roy (7). Application of this sequence of fractions was found to methylate both esterified fatty acids and nonesterified fatty acids. In these experiments, 17:0 was used as an internal standard. Alternatively, tripentadecanoin or triheptadecanoin was used as an internal standard when only the method of Turk *et al.* (10) was employed as this method is more efficient for esterified than for nonesterified fatty acids.

RESULTS

We first performed a series of nonesterified fatty acid methylations using the method of Lepage and Roy (7) without modification (Table 1). We collected data from 34 nonfasting subjects and 5 fasting subjects. These data were compared with those generated by Lepage and Roy (7), which were from one plasma sample analyzed five times. Table 1 shows that the concentrations of various nonesterified fatty acids found in fasting plasma were slightly higher than those in nonfasting plasma. This is consistent with reports that TG hydrolysis in the fasting state elevates nonesterified fatty acid concentrations (11). The concentration of nonesterified arachidonate we obtained using the unmodified Lepage and Roy (7) method in five specimens from fasting subjects was 36 μM, which

was very similar to the arachidonate concentration in the sample analyzed by Lepage and Roy (35.5 μM), and not significantly different from the 25 μM value we obtained for a group of 34 nonfasting individuals. To test whether the concentration of nonesterified arachidonate we obtained in both cases could have been an overestimation, we pursued the possibility that the unmodified Lepage and Roy (7) method may also lead to some methylation of bound fatty acid in the plasma. The cleavage and methylation of only a few percent of esterified fatty acid would, of course, greatly confound the measurement of the much smaller pool of nonesterified fatty acid. Table 2 shows the results of experiments in which we determined the percent cleavage of esterified fatty acids using the unmodified Lepage and Roy method. In these experiments we used TG, PL and CE, the amounts of which were known from the amounts of TG, PL and CE delivered from gravimetrically prepared stock solutions. In addition, the amounts of TG, PL and CE shown in the middle column of Table 2 were confirmed by using 248 nmol of trimyristin as internal standard followed by alkaline hydrolysis, methylation of the fatty acids and quantitation by gas chromatography (10). We tested two TG, two PL and two CE standards, one of each containing only saturated and the other only polyunsaturated fatty acids, to determine whether the degree of fatty acid unsaturation had an effect on fatty acid analysis by the unmodified Lepage and Roy (7) method. There were no free fatty acids detected in the TG, PL and CE standards used in these studies. The data, however indicate that there was indeed cleavage and methylation of esterified fatty acids. In plasma, the concentration of esterified fatty acids is more than 100-fold greater than the concentration of nonesterified fatty acids. Therefore, the 1.2–6.8% cleavage of esterified fatty acids which we observed represents a substantial amount of contamination relative to the pool of nonesterified fatty acid. We also found that esterified polyunsaturated fatty acids were more readily cleaved from TG and CE than were saturated fatty acids, but from PL saturated fatty acids were cleaved as readily as polyunsaturated ones. The significantly higher rate of cleavage of polyunsaturated fatty acids *vs.* saturated fatty acids from TG and CE indicates that the Lepage and Roy (7) method results in greater overestimation of polyunsaturated fatty acid than saturated fatty acid, particularly as TG in the plasma occur at substantial concentrations. Thus, when assaying by the Lepage and Roy method, the amount of contaminating fatty acid measured may actually exceed the mass of true nonesterified fatty acid.

We observed similar problems when we attempted to use the method of Pace-Asciak (8) for methylation of plasma nonesterified fatty acids without prior isolation (data not shown). A report demonstrating that the Pace-Asciak method overestimates plasma nonesterified fatty acid concentrations has recently been published (12). The values for plasma nonesterified fatty acids (nonfasting

TABLE 1

Nonesterified Fatty Acid Concentrations Determined by the Unmodified Lepage and Roy Method[a]

MeOH/acetyl chloride	50:1	50:1	50:1[b]
Fasting/nonfasting	Nonfasting	Fasting	Unknown
Number of samples	34	5	1
Fatty acid	μM Concentration		
16:0	182.6 ± 61.7	240.9 ± 56.3	260.5
18:0	70.4 ± 24.9	67.8 ± 7.2	109.7
18:1n-9	150.5 ± 60.9	211.0 ± 27.4	128.1
18:2n-6	178.2 ± 70.7	249.3 ± 47.7	176.5
20:4n-6	24.9 ± 11.0	36.3 ± 14.3	35.5
Total	606.5 ± 205.7	805.4 ± 134.8	709.4

[a]Nonesterified fatty acid concentrations in plasma samples from fasting and nonfasting subjects were determined using the unmodified method of Lepage and Roy (Ref. 7) (room temperature) as detailed in Materials and Methods. Values represent mean ± SD.
[b]The previously published values by Lepage and Roy are from one sample tested five times as reported in Reference 7.

TABLE 2

The Hydrolysis of Esterified Fatty Acids by the Unmodified Lepage and Roy Method[a]

Lipid	FA	Concentration of FA in solution (μM)	Concentration of FA methyl ester according to Ref. 7 (μM ± SEM)	% Ester cleavage
Triacylglycerol				
Tripentadecanoin	Saturated	6456	77.7 ± 5.5	1.2
Triarachidonin	Polyunsaturated	5192	218.5 ± 13.3	4.2
Phospholipid				
Dipalmitoyl PC	Saturated	4632	301.7 ± 10.3	6.5
Diarachidonoyl PC	Polyunsaturated	4095	231.7 ± 8.8	5.7
Cholesteryl ester				
Cholesteryl heptadecanoate	Saturated	2034	32.0 ± 2.1	1.6
Cholesteryl linoleate	Polyunsaturated	2005	135.4 ± 7.3	6.8

[a]Percent hydrolysis was calculated as 100 × (mean concentration of methylated fatty acids according to Lepage and Roy method, Ref. 7)/(concentration of esterified fatty acid in solution). FA, fatty acid; PC, phosphatidylcholine; n = 3 for all lipid moieties tested.

donors) obtained in our studies with the Pace-Asciak method (8) were: 16:0, 239 ± 7; 18:0, 113 ± 12; 18:1, 195 ± 26; 18:2, 153 ± 11; 20:4, 44 ± 4 (all values in μM; mean ± SEM, n=3). These values were very similar to those reported by Lepage and Roy and were also similar to our own results obtained by the Lepage and Roy (7) method (Table 1).

In an attempt to determine accurate and precise values for the individual nonesterified fatty acids in the plasma, including the polyunsaturated fatty acids, free of contamination from the esterified fatty acid pool, we compared the quantitation of nonesterified fatty acids by the Lepage and Roy method (at 0°C, which decreases cleavage of esterified fatty acid but does not eliminate it) with the quantitation of plasma nonesterified fatty acid following lipid extraction, isolation of NEFA by TLC, and methylation of NEFA using the procedure of Lepage and Roy (7) (Table 3). It is important to note that in these experiments, spotting of the lipid extract onto the TLC plate, drying of the developed TLC plate, identification of the bands on the plate and scraping the nonesterified fatty acid band from the TLC plate were all carried out in an atmosphere of nitrogen to prevent oxidation of polyunsaturated fatty acids. Also, to account for all losses of both saturated and unsaturated NEFA, due to oxidation and other causes, we used an internal standard mixture of four fatty acids with 0, 1, 2 or 4 double bonds (17:0, 20:1n-9, 20:2n-6 and 22:4n-6). Each of the internal standards, including the polyunsaturated ones, when used in the calculation of plasma nonesterified fatty acid concentrations yielded the same results, indicating that only an insignificant amount of polyunsaturated fatty acid oxidation, if any, occurred in our experiments. As shown in Table 3, the concentrations of the individual nonesterified fatty acids determined following isolation of plasma NEFA by TLC with precautions taken to avoid oxidation were significantly lower than the values obtained by Lepage and Roy (7). This was true not only for arachidonate, but also for the other plasma nonesterified fatty acids. A number of methods have been developed for the quantitation of plasma nonesterified fatty acids, most or all of which are less cumbersome than the reference method we used involving multiple internal standards and frequent use of nitrogen in isolation of the nonesterified fatty acids. However, these methods have generated values for plasma NEFA that vary over a very wide range. In view of the reproducibility of the method we used involving only conventional techniques, but avoiding shortcuts, and with the inclusion of multiple internal standards to account for various losses, it is likely that the data presented in Table 3 give the true NEFA concentrations in plasma. From the last column of Table 3, it can be seen that the concentrations of nonesterified fatty acids following isolation by TLC were 13–52% of the values obtained by the Lepage and Roy (7) method at 0°C. As suggested by our earlier results shown in Table 2, these data also show that the Lepage and Roy method overestimates polyunsaturated nonesterified fatty acids to a greater extent than nonpolyunsaturated fatty acids. The nonesterified arachidonic acid concentration measured following isolation by TLC was only 13% of the Lepage and Roy values. The concentration of nonesterified linoleic acid having two double bonds rather than four, was somewhat higher at 22%. Because the values for these two polyunsaturated fatty acids were lower relative to those for the more saturated fatty acids, the susceptibility of polyunsaturated fatty acid to oxidation during TLC was tested directly (Table 4). In the first two columns of Table 4, the plasma nonesterified fatty acid concentrations obtained by the unmodified Lepage and Roy (7) method on unfractionated plasma are shown. Only one μL of more than 100 μL of extracted plasma sample was injected into the gas chromatograph, and the remainder of the sample containing fatty acid methyl esters was subjected to TLC. The fatty acid methyl ester band was identified, and as in all other experiments, scraped from the plate in an atmosphere of nitrogen. The fatty acid methyl esters were then analyzed by gas chromatography. As noted by comparing the first and second columns of Table 4, the percentages for each individual fatty acids are essentially identical, for linoleic and arachidonic acid as well as for the other nonesterified fatty acids. This indicates that the additional thin-layer chromatographic step to separate the nonesterified fatty acids from TG, PL and CE did not result in oxidation of polyunsaturated fatty acids. We also performed other experiments to test for oxidation-induced

TABLE 3

Comparison of Nonesterified Plasma Fatty Acid Concentrations Determined by the Lepage and Roy Method on Unfractionated Plasma *vs.* Methylation of Isolated Nonesterified Fatty Acids[a]

NEFA source	Unfractionated plasma	Isolated from plasma by TLC	
MeOH/acetyl chloride	50:1	50:1	
Reaction temperature	0°C	RT	
Fasting/nonfasting	Nonfasting	Nonfasting	
Number of samples	6	5	
Fatty acid	μM Concentration	μM Concentration	% Of unfractionated plasma
16:0	86.3 ± 24.2	30.0 ± 5.4	35
18:0	28.4 ± 4.9	14.6 ± 1.7	52
18:1n-9	72.8 ± 11.6	27.8 ± 11.9	38
18:2n-6	83.1 ± 17.1	18.1 ± 4.4	22
20:4n-6	14.0 ± 5.6	1.8 ± 0.4	13
Total	284.6 ± 58.6	92.3 ± 21.1	32

[a]Samples of unfractionated plasma were processed for nonesterified fatty acid (NEFA) quantitation using the Lepage and Roy (Ref. 7) method at 0°C. Nonesterified plasma fatty acids isolated by TLC were also quantitated. Values represent mean ± SD; TLC, thin-layer chromatography; RT, room temperature.

TABLE 4

Fatty Acid Concentration Determined by the Lepage and Roy Method with or Without Prior Isolation of Nonesterified Fatty Acids by Thin-Layer Chromatography[a]

Fatty acid	Source of fatty acids			
	Plasma without TLC	Plasma with TLC	Standard mixture without TLC	Standard mixture with TLC
	(% of total fatty acids)			
16:0	28.5	28.1	26.2	26.2
18:0	9.5	9.7	7.9	8.3
18:1n-9	32.0	30.8	24.7	24.9
18:2n-6	26.7	27.6	35.9	35.3
20:4n-6	3.3	3.8	5.3	5.3
Total	100.0	100.0	100.0	100.0

[a]Plasma nonesterified fatty acids were methylated by the Lepage and Roy (Ref. 7) method, and the resultant fatty acid methyl esters split into two fractions; one fraction was purified by TLC, one was injected directly into the gas chromatograph. A standard mixture of fatty acid methyl esters was also split into two fractions and processed similarly. Values represent the mean of two determinations for each sample. TLC, thin-layer chromatography.

loss of polyunsaturated nonesterified fatty acids following isolation by TLC, the data for which are shown in columns 3 and 4 of Table 4. For these studies, we injected one μL of a standard fatty acid methyl ester mixture directly into the gas chromatograph. We then chromatographed the same standard fatty acid methyl ester mix on a thin-layer plate, isolated the fatty acid methyl ester band, eluted it and injected the eluted fatty acid methyl esters into the gas chromatograph. As shown in Table 4, this did not result in oxidation of linoleate and arachidonate.

Collectively, the data in Table 4 indicate that as we performed the experiments, there was no preferential loss of linoleate and arachidonate due to oxidation. In additional studies on 10 samples in which we did not take precautions to limit polyunsaturated fatty acid oxidation, TLC plates were allowed to sit exposed to room air for up to 1 h, and then NEFA bands were scraped from the plate in room air over 5–10 min. In all the 10 samples tested, no arachidonate was detected, and linoleate (as percent of total fatty acid) decreased from 25–35% to 5–10%. This clearly shows that isolation of NEFA by TLC before quantitation will lead to spuriously low values, unless simple precautions are taken to limit oxidation-induced losses.

Table 5 shows the reproducibility of the nonesterified fatty acid measurements following NEFA isolation by TLC when using multiple internal standards. In this series of experiments, we used the plasma from one subject and analyzed seven separate aliquots of the same specimen identically. The coefficients of variation were not large for any of the fatty acids, with the highest values of 10.2 and 10.7 observed for palmitoleic acid (16:1n-7) and arachidonic acid (20:4n-6). These two particular nonesterified fatty acids, because they occur at the lowest concentrations, showed the greatest variability in the measurements.

TABLE 5

Reproducibility of Nonesterified Fatty Acid Measurements Following Isolation by Thin-Layer Chromatography[a]

Fatty acid	NEFA μM concentration		
	Mean	SD	CV
14:0	3.2	0.2	7.6
16:0	40.1	1.9	4.7
16:1n-7	3.3	0.3	10.2
18:0	17.7	0.6	3.4
18:1n-9	40.7	3.2	7.9
18:2n-6	22.9	1.1	5.0
20:4n-6	1.6	0.2	10.7
Total	129.5	7.1	5.5

[a]Values represent the mean ± SD and include the coefficient of variation (CV) for measurement of total NEFA and each individual fatty acid isolated from seven aliquots of the same plasma. NEFA, nonesterified fatty acids.

DISCUSSION

We have demonstrated that using the method of Lepage and Roy (7) to quantitate plasma nonesterified fatty acids leads to significant cleavage of esterified fatty acids, which contaminates the nonesterified fatty acid pool and makes accurate measurements impossible. In a recent report, Lin *et al.* (12) provided evidence for extensive phospholipid fatty acid methylation that occurred during the assumed selective methylation of plasma free fatty acids by diazomethane (8). In the present study, using a method for quantitating nonesterified fatty acid not involving diazomethane, cleavage and methylation of fatty acids esterified to phospholipids, as well as to triglycerides and CE, was observed. In the Discussion section, Lin *et al.* (12) stated that in the process of evaluating in detail the diazomethane method developed by Pace-Asciak they also observed that the acetyl chloride/methanol method developed by Lepage and Roy (7) overestimates plasma nonesterified fatty acid levels. Lin *et al.* (12) did not support this conclusion with data. Because of the reluctance of many investigators to work with diazomethane, we assessed the acetyl chloride/methanol method in some detail. In our evaluation, we quantitated the extent of overestimation for various types of plasma nonesterified fatty acids and demonstrated the preferential cleavage of esterified polyunsaturated fatty acids from TG and CE, but not from PL. Thus, this report and the one by Lin *et al.* (12) provide quantitative evidence that two popular methods for the assumed selective methyla-

tion of nonesterified fatty acids lead to overestimation of the small pool of plasma free fatty acids. The method we used for comparison with the Lepage and Roy (7) method involved isolation of the nonesterified fatty acids prior to methylation, with precautions being taken to limit fatty acid oxidation and to quantitate any losses due to any oxidation. The present method may be somewhat more cumbersome than the two direct methods, but it provides accurate and precise measurements of the free fatty acid concentrations in plasma.

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Profiling of Arachidonic Acid Metabolites in Rabbit Platelets by Radio Gas Chromatography

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A method for profiling arachidonic acid metabolites by radio gas chromatography (GC) is described. The incubation mixture of rabbit platelets with [^{14}C]arachidonic acid was purified on a Sep-Pak C_{18} cartridge and derivatized with diazomethane, *O*-methylhydroxylamine and dimethylisopropylsilylimidazole. The recovery of total ^{14}C-radioactivity was 93.1 ± 7.2%. Loss of radioactivity during derivatization was negligible. Baseline separations for [^{14}C]arachidonic acid and its metabolites were obtained in a single run within 45 min by GC using a synchronized accumulating radioisotope detector (GC/SARD). The recovery of radioactivity from the GC column was virtually 100%. The chemical structures of the metabolites were confirmed by GC/mass spectrometry; peaks of arachidonic acid metabolites were assigned by comparison of the methylene unit values with those of radioactive peaks in GC/SARD analyses. The intra-assay coefficients of variation in GC/SARD analyses were less than 10%. The method was used to map the profile of arachidonic acid metabolites formed by rabbit platelets in the presence of indomethacin, baicalein or glutathione.
Lipids 28, 361–364 (1993).

Arachidonic acid (AA) is enzymatically transformed into a variety of oxygenated products (eicosanoids) *via* two main pathways (1). The cyclooxygenase pathway leads to unstable endoperoxides that can be further converted to prostaglandins (PG) and thromboxanes (TX). The lipoxygenase pathway leads to hydroperoxyeicosatetraenoic acids (HPETE), hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT). Eicosanoids have a diverse range of physiological and pharmacological effects, and sometimes opposing effects; for example, TXA_2 stimulates (2), but PGD_2 (3), PGI_2 (4) and 12-HPETE (5) inhibit platelet aggregation.

Recently there have been numerous attempts to correlate the anti-inflammatory, antipyretic and analgesic effects of substances like aspirin, indomethacin and phenylpropionic acid derivatives with their ability to inhibit the production of AA metabolites in order to investigate the mechanism of action of these drugs (6–8). It is highly desirable to examine and quantitate as broad a band of AA metabolites as possible.

Radioisotope tracer techniques have been applied to investigate the profile of eicosanoids formed from AA. Thin-layer chromatography (TLC) (9–11) and high-performance liquid chromatography (HPLC) (12–16) with radiodetection have been used for the separation of radioisotopically labeled eicosanoids. The TLC methods do not provide sufficient resolution. The HPLC methods offer the advantage of high resolution and good reproducibility, but require long analysis times and a large amount of scintillation fluid. Although gas chromatography (GC) is generally accepted as one of the most effective methods for the separation of eicosanoids due to its excellent resolving power, GC combined with radiodetection (radio GC) has not been used previously for profiling complex mixtures of eicosanoids. Radio GC has the inherent drawback (17) that enhancement in detection sensitivity results in decreased resolution; this has lowered the usefulness of the technique. We have developed a radio GC system that utilizes a synchronized accumulating radioisotope detector (SARD) (18–20). In the SARD, seven identical counting tubes are connected in series so that signals from each counting tube are accumulated synchronously with the traveling radioactive gas particles. Moreover, the counting tubes are connected electrically by a mutual anti-coincidence circuit so that the background counts are effectively lowered and signal-to-noise ratios are improved tenfold without a loss in chromatographic resolution. SARD was successfully linked with capillary GC (21). The present paper describes a procedure for assessing the profile of eicosanoids formed from AA using [^{14}C]AA and capillary GC/SARD. Rabbit platelet homogenates were used as a model system for developing the procedure because they are readily harvested and they actively metabolize AA both through the cyclooxygenase and lipoxygenase pathways.

MATERIALS AND METHODS

Reagents. [1-^{14}C]AA (specific activity, 2.06 GBq/mmol; radiochemical purity, 99.2%) and 12-(*S*)-hydroxy[5,6,8,9,11,12,14,15(*n*)-[^{3}H]eicosatetraenoic acid ([^{3}H]12-HETE; specific activity, 5.29 TBq/mmol; radiochemical purity, 96.4%) were purchased from the Radiochemical Centre (Amersham, United Kingdom). Nonlabeled AA was purchased from Funakoshi (Tokyo, Japan). 12-HETE, 12-hydroxyheptadecatrienoic acid (12-HHT) were purchased from Sigma (St. Louis, MO). TXB_2 was kindly supplied by Upjohn Pharmaceutical Limited (Ibaraki, Japan). Dimethylisopropylsilyl(DMiPS)imidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). *O*-Methylhydroxylamine·HCl pyridine solution was purchased from Pierce (Rockford, IL). A Sep-Pak C_{18} cartridge was purchased from Waters Associates (Milford, MA). Indomethacin and baicalein were purchased from Wako (Tokyo, Japan). Glutathione (GSH) was purchased from Kanto Chemical (Tokyo, Japan).

Isolation of rabbit platelets. Fresh arterial blood (*ca.* 100 mL) was collected from a Japanese white rabbit into plastic tubes with 1/10 vol of 3.8% sodium citrate and centrifuged at 140 × *g* for 15 min to separate the platelet-rich plasma. The platelet-rich plasma was further centrifuged at 750 × *g* for an additional 15 min. The platelet pellet obtained from 50 mL of platelet-rich plasma was

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Abbreviations: AA, arachidonic acid; DMiPS, dimethylisopropylsilyl; EDTA, ethylenediaminetetraacetic acid disodium salt; EPHETA, 11,12-epoxy-10-hydroxyeicosa-5,8,14-trienoic acid; FID, flame ionization detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GSH, glutathione; HETE, hydroxyeicosatetraenoic acid; 12-HHT, 12-hydroxyheptadecatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; LT, leukotriene; MU, methylene unit; PG, prostaglandin; SARD, synchronized accumulating radioisotope detector; TLC, thin-layer chromatography; TX, thromboxane; 8,9,12-THETE, 8,9,12-trihydroxyeicosa-5,10,14-trienoic acid; 8,11,12-THETE, 8,11,12-trihydroxyeicosa-5,9,14-trienoic acid.

washed in 100 mL of 12 mM Tris-HCl buffer (pH 7.4) containing 134 mM NaCl and 1.5 mM ethylenediaminetetraacetic acid disodium salt (EDTA), and centrifuged at 750 × *g* for 15 min. After a second wash in the same buffer, the final pellet was suspended in 15 mM Tris-HCl buffer (pH 7.4) containing 134 mM NaCl and 5 mM D-glucose at a concentration of 3–5 × 10^5 platelets/μL. The washed platelet suspension was stored at −80°C.

Sample preparation. Frozen platelets were thawed and homogenized by triplicate sonication at 27 kHz for 1 min. One mL of the homogenate (*ca.* 1 mg protein/mL) was incubated at 37°C for 5 min with [^{14}C]AA (10 nmol, 20 kBq). The reaction was terminated by the addition of 0.2 mL of 0.2 M citric acid (pH 3). The reaction mixture was applied to a Sep-Pak C_{18} cartridge which was washed with 5 mL each of methanol and water immediately before use. The cartridge was washed with 10 mL each of 5% ethanol/water, water, and petroleum ether and then eluted with 6 mL of methyl formate. The eluate was evaporated to dryness under a nitrogen stream. The residue was derivatized with diazomethane, *O*-methylhydroxylamine and DMiPS-imidazole as described previously (22). The derivatized sample was dissolved in 50 μL of 1% pyridine/hexane, and 1–3 μL was injected into the GC/SARD system.

Samples for measurements by GC/MS were prepared as follows. Five mL of the rabbit platelet homogenate was incubated with 50 nmole of unlabeled AA and the incubation products were purified and derivatized as described above. The derivatized sample was dissolved in 50 μL of 1% pyridine/hexane, and 0.5–1 μL was injected into the GC/mass spectrometry (MS) system.

Radio GC system. All radio GC analyses were performed on the GC/SARD system described previously (21). A wide-bore fused silica capillary column (12 m × 0.53 mm) coated with Shimadzu (Kyoto, Japan) nonpolar CBP-1 (1 μm film thickness) was used. The flow rates of helium were 5 mL/min for carrier gas and 50 mL/min for make-up gas. The column effluents were subjected to oxidation ($^{14}C \rightarrow {}^{14}CO_2$) or oxidation/reduction ($^{3}H \rightarrow {}^{3}H_2O \rightarrow {}^{3}H_2$) in the oxidation/reduction tube, and the resulting gas was introduced into the SARD after mixing with counting gas (methane). In the SARD, the seven gas flow proportional counting tubes (inner volume, 10 mL) are arranged in close packing and placed in a 30-mm thick cylindrical lead housing. The counting tubes are connected by a mutual anti-coincidence circuit. The total flow rates of methane and helium in the SARD were maintained at 200 mL/min. The sampling time (the transit time of the gas particles through one counting tube) (21) was set at 3 s, and the transit time of gas particles in the SARD system was 21 s (7 × 3 s). The column oven temperature was set at 180°C and increased at a rate of 2°C/min to 270°C.

Peak radioactivities, A (Bq), were calculated according to the following equation.

$$A\ (Bq) = \frac{\text{radioactive peak intensity (counts)} \times 100}{\text{transit time in the SARD (s)} \times \text{GC peak yield (\%)}} \quad [1]$$

GC peak yields (percentage of the amount of an injected substance that reaches the detector system) were calculated by analyzing authentic [^{14}C]AA (120 Bq) or [^{3}H]12-HETE (120 Bq), according to the method in our previous papers (22,23).

GC/MS. GC/MS analyses were performed on a Shamadzu gas chromatograph model GC-14A equipped with a capillary column (10 m × 0.25 mm) coated with Supelco (Bellefonte, PA) nonpolar SPB-1 (film thickness, 0.25 μm), coupled directly to the source of Shimadzu QP-1000 EX mass spectrometer. Helium was used as carrier gas at a pressure of 0.5 kg/cm^2. The column oven temperature was maintained at 100°C for 1 min after injection and then increased at a rate of 10°C/min to 270°C. The ion source temperature was set at 270°C. A splitless injection mode was used, and the vent was opened at 1 min after injection. Mass spectra were recorded in the electron impact mode at an electron energy of 70 eV and a trap current of 60 μA. The mass spectra were recorded, covering an *m/z* range of 50–800 every 2 s.

Liquid scintillation counter. The radioactivities were measured in 10 mL of toluene base scintillation cocktail with an Aloka (Tokyo, Japan) liquid scintillation counter (LSC, 903 or 1000).

RESULTS AND DISCUSSION

The gas chromatographic analysis of eicosanoids has been described in several papers (24–27). Maclouf and Rigaud (24) reported the complete resolution of some eicosanoids by capillary column GC using a flame ionization detector (FID). However, this method is not suitable for profiling AA metabolites in biological samples due to the presence of several peaks on the GC tracing other than those from the analytes. The radio GC approach described here provided much clearer profiling as radiodetection is not obstructed by endogenous compounds and derivatizing reagents.

Sep-Pak C_{18} cartridges have been extensively used to separate AA metabolites from biological samples. The separation with Sep-Pak C_{18} cartridges utilized in the present study was essentially the same as that proposed by Powell (28). [^{14}C]AA and its metabolites were eluted with methyl formate with a recovery of 93.1 ± 7.2% (n = 10). Loss of radioactivity during derivatization was negligible.

A typical radiochromatogram obtained by incubation of rabbit platelet homogenate with [^{14}C]AA is shown in Figure 1. The recovery of radioactivity from the GC column was virtually 100%. Baseline separations for all major products from platelets were obtained within 45 min under linear temperature programing conditions. The

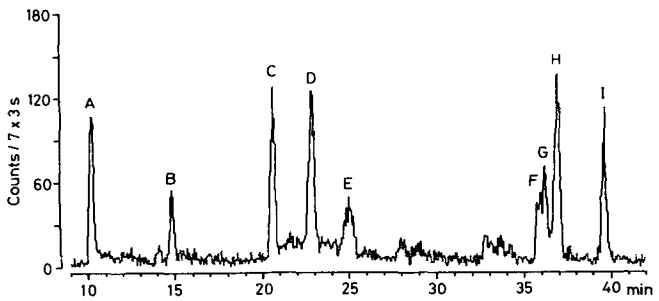

FIG. 1. Profile of [^{14}C]eicosanoids and its metabolites in rabbit platelets. Column, nonpolar fused silica capillary column (12 m × 0.53 mm); temperature programming, 180–270°C at 2°C/min; injected radioactivity, 514 Bq.

DMiPS derivatives were shown to be well separated and more stable than the corresponding trimethylsilyl derivatives.

Radioactive peaks in GC and HPLC are usually tentatively identified by comparing the retention times with those of reference standards. However, this approach is often ambiguous. In the present study, an attempt was made to directly confirm the structures of the metabolites by GC/MS analyses. The incubation mixture with unlabeled AA was extracted and derivatized in a procedure similar to that used for labeled AA and was analyzed by GC/MS. Each peak in GC/MS analysis was assigned by comparison of the methylene unit (MU) (29,30) values with those of the radioactive peaks in GC-SARD analysis. The metabolites in peaks A (MU; 22.2), B (MU; 24.0), C (MU; 26.0) and I (MU; 23.5) were identified as AA, 12-HHT, 12-HETE and TXB_2, respectively, by comparison with the mass spectra with those of authentic compounds. The metabolite in peak D (MU; 26.0) was identified as 11,12-epoxy-10-hydroxyeicosa-5,8,14-trienoic acid (EPHETA). Its mass spectrum showed the major fragment ions at *m/z*: 419[M − 31, loss of OCH_3], 407[M − 43, loss of $CH(CH_3)_2$], 310[M − 140, loss of $OHCCH_2CH{=}CH(CH_2)_4CH_3$], 297[M − 153, loss of $CH(O)CHCH_2CH{=}CH(CH_2)_4CH_3$], 279[M − (140 + 31)], 267[M − (140 + 43)], 265 [M − (111 + 31 + 43), loss of (C-13 to C-20), OCH_3 and $CH(CH_3)_2$], 221[M − (111 + 118), loss of (C-13 to C-20) and DMiPSOH]. The mass spectra of peaks F (MU; 31.2),

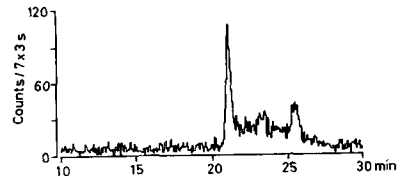

FIG. 2. Radio gas chromatogram of [^{3}H]12-hydroxyeicosatetraenoic acid after treatment with diazomethane, *O*-methylhydroxylamine and dimethylisopropylsilyl-imidazole. See Figure 1 for gas chromatographic conditions. Injected radioactivity, 100 Bq.

TABLE 1

Reproducibility of Analyses of [^{14}C]Arachidonic Acid (AA) and Its Metabolites

Peak[a]	Metabolite[b]	% of Injected radioactivity[c]	Coefficient of variation (%)
A	AA	7.1 ± 0.04	0.6
B	12-HHT	3.1 ± 0.26	8.4
C+E	12-HETE	18.4 ± 0.94	5.1
D	EPHETA	8.6 ± 0.02	0.2
F+G+H	THETEs	18.6 ± 0.34	1.8
I	TXB_2	6.6 ± 0.25	3.8

[a]Peaks designated by letter refer to Figure 1.
[b]12-HHT, 12-hydroxyheptadecatrienoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; EPHETA, 11,12-epoxy-10-hydroxyeicosa-5,8,14-trienoic acid; THETE, trihydroxyeicosa-5,9,14-trienoic acid; TXB_2, thromboxane B_2.
[c]514 Bq (n = 3).

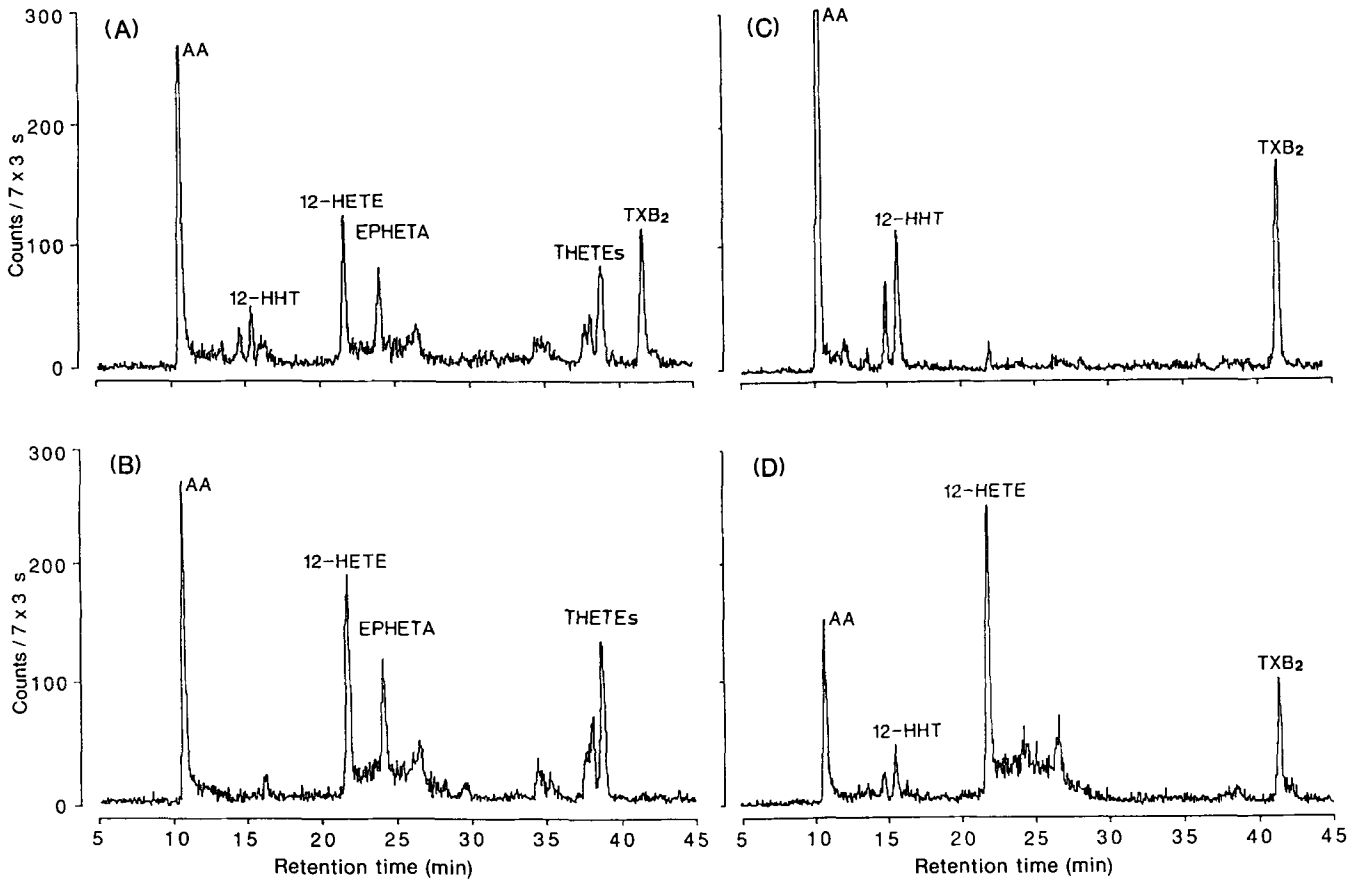

FIG. 3. Effects of inhibitors on [^{14}C]AA metabolism. One mL of platelets homogenate (*ca.* 1 mg protein/mL) was preincubated for 5 min with inhibitors (added in 1 μL ethanol) prior to addition of [^{14}C]AA (10 nmol, 8 kBq). Other procedures were as described in the Experimental section. Injected radioactivity, 300–500 Bq; (A), control; (B), indomethacin (56 μM); (C), baicalein (10 μM); (D), GSH (1 mM). Abbreviations as in Table 1.

G (MU; 31.3) and H (MU; 31.6) were almost identical. The metabolites reflected in these peaks were identified as a mixture of stereoisomers of 8,9,12-trihydroxyeicosa-5,10, 14-trienoic acid (8,9,12-THETE) and 8,11,12-THETE because the mass spectra showed the characteristic fragment ions at *m/z* 241 and 271 with similar intensity; this means preferential fragmentation occurs between the two adjacent DMiPS-substituted hydroxyl groups in 8,9,12- and 8,11,12-THETEs, respectively.

Thermal decomposition on a GC column has been noted in the analysis of HETEs (31,32). Some decomposition for 12-HETE was also observed in our investigation. Figure 2 shows a radiochromatogram of authentic [^{3}H]12-HETE after derivatization. The main peak (t_R 21.1 min; MU 26.1) is followed by a slightly elevated background and then by the second peak (t_R 25.5 min; MU 27.7). The MU value of the second peak was identical to that of peak E in Figure 1.

The counting reproducibility of the GC/SARD system was determined by repeated injections (n = 3). Each peak radioactivity was calculated based on the GC peak yields. The GC peak yields of the metabolites, except for 12-HETE, were assumed to be equal to that of AA (22). The GC peak yield of 12-HETE was 34.2% (coefficient of variation 5.7%, n = 3). The intra-assay coefficients of variation in GC/SARD analyses were less than 10% (Table 1).

Inhibition studies were performed in order to confirm the validity of the present method. [^{14}C]AA was incubated with platelets in the presence of indomethacin (56 μM), baicalein (10μM) or GSH (1 mM). Examples of the radiochromatograms obtained are shown in Figure 3. Indomethacin inhibited cyclooxygenase and eliminated 12-HHT and TXB_2. Baicalein, a selected inhibitor of 12-lipoxygenase, inhibited formation of 12-HETE, EPHETA, 8,9,12-THETE and 8,11,12-THETE. GSH inhibited formation of EPHETA, 8,9,12-THETE and 8,11,12-THETE but increased formation of 12-HETE by GSH-dependent peroxidase (33,34).

The present capillary GC/SARD approach provides a useful method for profiling AA metabolites and can also be used to detect inhibitors of AA metabolism.

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ARTICLE

Dependence of the Bilayer Phase Transition Temperatures on the Structural Parameters of Phosphatidylcholines

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Most saturated diacyl phosphatidylcholines C(X):C(Y)PC (saturated 1,2-diacyl-*sn*-glycero-3-phosphocholine with X carbons in the *sn*-1 acyl chain and Y carbons in the *sn*-2 acyl chain), in excess water, can self-assemble into lamellae which, upon heating, may undergo multiple thermotropic phase transitions at well-defined, discrete temperatures. The transition temperature corresponding to the main or the gel to liquid-crystalline phase transition (T_m) is known for many bilayers of fully hydrated phosphatidylcholines. In this study, we have analyzed the T_m values of 44 molecular species of phosphatidylcholines in terms of their structural and packing characteristics in the gel-state bilayer. Two general equations are thus derived: $T_m = 162.26 - 3651.71\ (1/N) - 88.42\ (\Delta C/N)$ for C(X):C(Y)PC with $X \geqslant Y$, and $T_m = 157.68 - 3525.44\ (1/N) - 93.28\ (\Delta C/N)$ for C(X):C(Y)PC with $X < Y$. Here, N is the minimal hydrophobic thickness of the dimeric C(X):C(Y)PC in the gel-state bilayer and ΔC is the effective chain length difference between the *sn*-1 and *sn*-2 acyl chains for the monomeric C(X):C(Y)PC in the gel-state bilayer. The advantage of these two equations in predicting the T_m values for phosphatidylcholines with ΔC/CL values in the range of 0.07 to 0.40 is their simplicity. A figure containing a total of 173 calculated T_m values is also presented.
Lipids 28, 365–370 (1993).

A fundamental and important concept of biochemistry is that the structural characteristics of biomolecules existing as either monomers or aggregated assemblies are ultimately responsible for their physical and chemical properties in the cell and in aqueous solution, under physiological conditions. Of the many physical properties associated with the main or the gel to liquid-crystalline phase transition for phospholipid bilayers in aqueous solution, the phase transition temperature (T_m) is the one that can be most accurately determined experimentally (1–3). The T_m values for aqueous lipid dispersions prepared from saturated, identical-chain phosphatidylcholines have been shown by several groups (4,5) to be related to certain structural features of the lipid molecules, such as the acyl chain length and the chain end perturbation (6,7). More recently, the structure-property relationship has been extended further (8) to include mixed-chain phosphatidylcholines, thus allowing the accurate prediction of T_m values from the structural characteristics expressed by a large number of phosphatidylcholines. The general equation of Huang (8), $T_m = 154.2 + 2.0\ (\Delta C) - 142.8\ (\Delta C/CL) - 1512.5\ (1/CL)$, has four terms with three variables (ΔC, ΔC/CL and 1/CL); moreover, these variables are related to two structural parameters (ΔC and CL) which are determined by the conformations of the two acyl chains of a monomeric phosphatidylcholine molecule in the gel-state bilayer. Here, the structural parameter ΔC denotes the effective chain length difference (in C-C bonds lengths along the chain) between the two acyl chains, and the other structural parameter, CL, is the effective length of the longer of the two acyl chain, also in C-C bonds (9,10), as shown in Figure 1A.

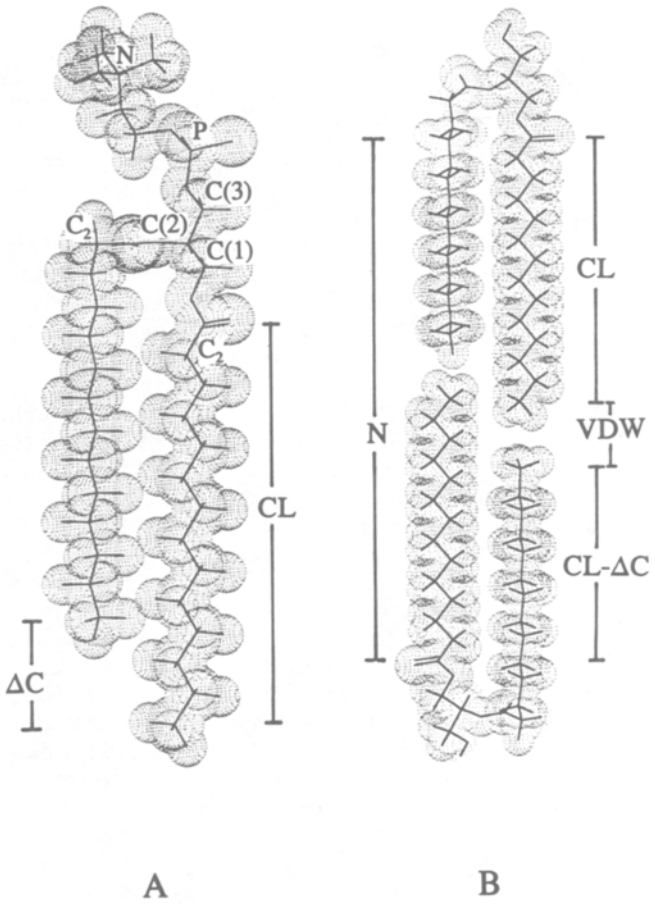

FIG. 1. Computer-generated diagrams to illustrate the acyl chain packing in (A) single crystal of C(14):C(14)PC, (B) a dimeric packing unit of C(X):C(Y)PC (saturated 1,2-diacyl-*sn*-glycero-3-phosphocholine with X carbons in the *sn*-1 acyl chain and Y carbons in the *sn*2 acyl chain) constructed based on the monomeric structure of (A). Only the diglyceride moiety of the lipid molecule is drawn in (B). ΔC is the effective chain length difference between the two chains in a monomeric lipid molecule. It equals 1.5 C-C bond lengths for C(14):C(14)PC in the gel-state bilayer. CL is the effective length of the longer of the two acyl chains. N is the distance separating the two carbonyl oxygens of the *sn*-1 acyl chains. VDW is the Van der Waals distance separating the two methyl termini.

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Abbreviations: C(X):C(Y)PC, saturated 1,2-diacyl-*sn*-glycero-3-phosphocholine with X carbons in the *sn*-1 acyl chain and Y carbons in the *sn*-2 acyl chain; MW, molecular weight; RMSE, root mean square error; T_m, the phase transition temperature; VDW, Van der Waals distance.

The ratio of ΔC/CL is thus the normalized chain length difference for phosphatidylcholines in the highly ordered gel state.

In this communication, alternative equations are presented for predicting the T_m values for fully hydrated saturated phosphatidylcholines which can undergo the gel to liquid-crystalline phase transition upon heating. In this new approach, the transition temperature is also related to two structural parameters (N and ΔC). These equations, however, are simpler, having only three terms. Moreover, the predictive power is also improved in comparison with that of the general equation of Huang (8).

RESULTS

The structural parameters: ΔC, ΔC/CL and N. Prior to defining the various structural parameters, it is appropriate to briefly mention the structural characteristics of the two acyl chains in dimyristoyl phosphatidylcholine, C(14):C(14)PC. This is an identical-chain phosphatidylcholine whose crystal structure has been determined by X-ray diffraction technique (11). In single crystals, the C(1) and C(2) carbon atoms of the glycerol backbone and the fatty acyl chain ester-linked to the *sn*-1 position of the glycerol C(1) atom form an all *trans* zigzag chain (Fig. 1A). The initial segment of the *sn*-2 acyl chain, however, extends perpendicularly from the *sn*-1 acyl chain, but bends over at the second carbon atom, C_2, to become parallel to the *sn*-1 acyl chain, as schematically illustrated in Figure 1A. Moreover, the orientation of the two chain planes within each lipid molecule is alternately parallel and perpendicular to each other in the bilayer assembly. In Figure 1A, the two zigzag planes of the *sn*-1 and *sn*-2 acyl chains of a C(14):C(14)PC molecule are shown to be perpendicular to each other. Another structural feature of the two acyl chains, as illustrated in Figure 1A, is that the two terminal methyl groups are mismatched. This mismatch is defined as the effective chain length difference (ΔC) between the two acyl chains which, for C(14):C(14)PC in the crystalline state, is about 3.7 Å or 3.0 carbon-carbon bond lengths along the long chain axis. The two acyl chains are tilted with an angle of about 12°C relative to the axis perpendicular to the bilayer surface. For identical-chain C(14):C(14)PC in the gel-state bilayer, the structural features of the lipid acyl chains observed in the single crystals are essentially preserved; however, the chains are tilted more with an angle of about 30°C (12) and the axial displacement of the two methyl termini along the bilayer normal is about 1.5 carbon-carbon bond lengths (13).

The structural parameter ΔC is defined for a C(X):C(Y)PC (saturated 1,2-diacyl-*sn*-glycero-3-phosphocholine with X carbons in the *sn*-1 acyl chain and Y carbons in the *sn*-2 acyl chain) molecule in the gel-state bilayer based on the inherent mismatch of 1.5 C-C bond lengths between the two terminal methyl groups in the monomeric C(14):C(14)PC as follows: $\Delta C = |(X - 1) - (Y - 1 - 1.5)| = |X - Y + 1.5|$, where X and Y are the total numbers of carbon atoms in the saturated *sn*-1 and *sn*-2 acyl chains, respectively. The normalized chain length difference between the *sn*-1 and *sn*-2 acyl chain for a C(X):C(Y)PC molecule in the gel-state bilayer is expressed as ΔC/CL, where CL is the effective length of the longer of the two acyl chains in C-C distance along the chain.

When two phosphatidylcholine molecules are packed in the partially interdigitated mode, as shown in Figure 1B, a structural parameter, N, can be employed to specify the distance between the two carbonyl oxygens of the *sn*-1 acyl chains in the two opposing bilayer leaflets. This distance is tantamount to the minimal hydrophobic thickness of a dimeric unit of phosphatidylcholine molecules packed in the gel-state bilayer, and it can be expressed in C-C bond lengths along the long molecular axis of the lipid. As shown in Figure 1, the structural parameter, N, of the dimeric unit is related to the two structural parameters of the monomer (ΔC and CL) as follows: $N = 2CL - \Delta C + VDW$. For C(X):C(Y)PC with $X \geq Y$, it can easily be shown that the value of N is related to X and Y as follows: $N = 2(X\text{-}1) - (X\text{-}Y + 1.5) + VDW$ (Van der Waals distance) $= X + Y - 0.5$, where the VDW between the two opposing terminal methyl groups is assumed to be three C-C bond lengths along the chain. In fact, this equation of $N = X + Y - 0.5$ is general for all gel-state phosphatidylcholines, C(X):C(Y)PC, which are packed in the partially interdigitated mode.

The main phase transition temperature as a function of lipid structural parameters. In the past three years we have examined the thermotropic phase behavior of aqueous dispersions prepared from four series of saturated diacyl phosphatidylcholines by high-resolution differential scanning calorimetry (14-16). Within each series, all members have a common molecular weight (MW); however, the values of ΔC/CL are varied progressively from 0.05 to 0.67. When the T_m (or ΔH) values of these four series of lipids are plotted against the normalized acyl chain length difference, biphasic curves as shown in Figure 2 are observed. We find that in the T_m (or ΔH) *vs.* ΔC/CL plot, the T_m (or ΔH) value of the lipid dispersion within each of the four series decreases initially with increasing chain asymmetry. In fact, within the range of ΔC/CL values of 0.09–0.40, the experimental T_m values appear to fall on a linear curve. The minimum value of T_m within each lipid series corresponds to the phosphatidylcholine molecule with a ΔC/CL value of about 0.41. Beyond the minimum value, the T_m value increases with increasing value of ΔC/CL; moreover, the data fall on a bell-shaped curve with the maximum value of T_m at ΔC/CL of about 0.56. A detailed interpretation of the biphasic phenomenon is given elsewhere (15,16).

All the published calorimetric T_m values for saturated diacyl phosphatidylcholines with ΔC/CL values ranging from 0.07 to 0.40 are summarized in Table 1. There are 44 data points, 24 of them belong to the four homologous series of lipids labelled as the C(14):C(14)PC, C(15):C(15)PC, C(16):C(16)PC and C(17):C(17)PC series. The T_m values of these 24 lipids are plotted in Figure 3 against ΔC/N. Clearly, within each series, the T_m values of lipids with longer *sn*-2 acyl chains can fit into a curve which lies slightly but consistently below the T_m curve for lipids with longer *sn*-1 acyl chains. The difference in T_m, albeit small, can be assumed to arise from the possibility that the lipid-lipid interaction for C(X):C(Y)PC with $X > Y$, in the gel-state bilayer, is somewhat different from that for C(X):C(Y)PC with $Y > X$.

The dependence of T_m on structural parameters. An interesting feature of the experimental data shown in Figure 3 is that the T_m values for various C(X):C(Y)PC with constant values of MW are linear functions of ΔC/N.

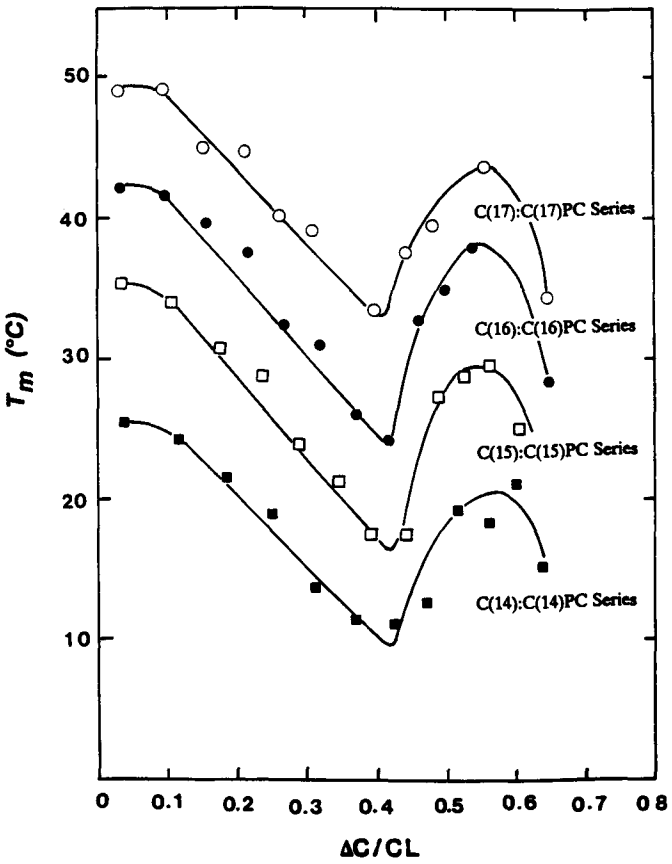

FIG. 2. Plot of the main phase-transition temperature (T_m) *vs.* the normalized chain length difference (ΔC/CL) between the two acyl chains of various phosphatidylcholines. The various phosphatidylcholines are grouped into four homologous series according to their molecular weight (MW), and the MW of the four series correspond to those of C(17):C(17)PC, C(16):C(16)PC, C(15):C(15)PC and C(14):C(14)PC, respectively.

Similarly, linear lines fitting T_m values are observed at constant ΔC values in the T_m *vs.* ΔC/N plot. These linear property-structure relationships suggest that the T_m value of the C(X):C(Y)PC bilayer may depend on the interplay of the two structural parameters ΔC and N. That is, $T_m = a_0 + a_1\,(1/N) + a_2\,(\Delta C/N) + \ldots$ might give a good fit to all T_m data obtained from aqueous dispersions of C(X):C(Y)PC with X > Y or with X < Y, where a_0, a_1 and a_2 are constants.

When the 22 experimentally observed T_m values of C(X):C(Y)PC with X ⩾ Y, shown in Table 1 (points Nos. 1-22), are subjected to statistical analysis using a multiple regression approach, a regression equation of $T_m = 162.26 - 3651.71\,(1/N) - 88.42\,(\Delta C/N)$ is obtained, with a correlation coefficient (σ) of 0.9996 and a root mean square error (RMSE) of 0.4791. Similarly, a regression equation of $T_m = 157.68 - 3525.44\,(1/N) - 93.28\,(\Delta C/N)$ with $\sigma = 0.9995$ and RMSE = 0.5332 is obtained for C(X):C(Y)PC with X < Y, when the other 22 T_m values from Table 1 (points Nos. 23-44) are subjected to multiple regression analysis.

The calculated and the experimental T_m values for all 44 molecular species of phosphatidylcholines are compared and the results are summarized in Table 1. Within each set of the data, only one lipid exhibits a deviation of greater than 1°C between the calculated and the observed T_m values (ΔT_m). The greatest deviation is observed for C(17):C(15)PC. This deviation, 1.2°C, corresponds to a relative error of 3.3% in °C or a relative error of 0.4% in absolute temperature. Because of the excellent agreement between the calculated and experimental T_m values, the two regression equations, $T_m = 162.26 - 3651.71\,(1/N) - 88.42\,(\Delta C/N)$ for C(X):C(Y)PC with X ⩾ Y and $T_m = 157.68 - 3525.44\,(1/N) - 93.28\,(\Delta C/N)$ for C(X):C(Y)PC with X < Y, can be used reliably to predict the T_m values for C(X):C(Y)PC with ΔC/CL values in the range of 0.07-0.40. Consequently, a table containing a total of 173 predicted T_m values is presented in Figure 4.

DISCUSSION

It has long been recognized that in excess water all saturated diacyl phosphatidylcholines with long acyl chains exhibit unique transition characteristics upon heating, and that the main phase transition temperature (T_m) increases with increasing chain length, all other things being equal (17,18). Recently we have examined and analyzed the structure-property relationship for a large number of saturated diacyl phosphatidylcholines C(X):C(Y)PC; we showed that the T_m values for various C(X):C(Y)PC with ΔC/CL values in the range of 0.09-0.40 can be related to the structural parameters ΔC and CL as follows (8): $T_m = 154.2 + 2.0\,(\Delta C) - 142.8\,(\Delta C/CL) - 1512.5\,(1/CL)$. This general equation of Huang's (8) was derived based on (i) the statistical analysis of T_m values obtained with 23 molecular species of C(X):C(Y)PC; and (ii) the structural parameters (ΔC and CL) associated with the monomeric unit of C(X):C(Y)PC in the gel-state bilayer. Although the predictive power of the general equation of Huang (8) is very high, this equation is cumbersome, having four terms and three variables (ΔC, ΔC/CL and 1/CL).

More experimental T_m values for fully hydrated C(X):C(Y)PC with ΔC/CL values in the range of 0.07-0.40 have been published (17,18); hence, the data base for our statistical analysis can be expanded to include 44 T_m values. Together with the knowledge of the proposed dimeric packing model for C(X):C(Y)PC in the gel-state bilayer as given in Figure 1B, we now derive the following two equations:

For C(X):C(Y)PC with X ⩾ Y

$$T_m = 162.26 - 3651.71\,(1/N) - 88.42\,(\Delta C/N) \quad [1]$$

with $\sigma = 0.99962$, RMSE = 0.47910

For C(X):C(Y)PC with X < Y

$$T_m = 157.68 - 3525.44\,(1/N) - 93.28\,(\Delta C/N) \quad [2]$$

with $\sigma = 0.99951$, RMSE = 0.53319

where σ is the correlation coefficient.

The major advantage of these two new equations is their simplicity. Each equation has only three terms with two variables (1/N and ΔC/N). A single equation can also be derived to fit all 44 data points. This equation, $T_m = 158.67 - 3532.18\,(1/N) - 94.99\,(\Delta C/N)$ with $\sigma = 0.99883$ and RMSE = 0.83735, is less accurate in predicting T_m due to its smaller value of σ and larger value of RMSE.

TABLE 1

The Main Phase Transition Temperatures (T_m) for Saturated Diacyl Phosphatidylcholines with ΔC/CL Values in the Range of 0.07–0.40[a]

No.	Lipid	ΔC	1/N	T_m^{obs}	T_m^{cal}	ΔT_m	Reference
1	C(13):C(13)PC	1.5	0.0392	13.9	13.9	0.0	up[b]
2	C(14):C(14)PC	1.5	0.0364	24.1	24.6	−0.5	15
3	C(15):C(15)PC	1.5	0.0339	34.0	34.0	0.0	15
4	C(16):C(16)PC	1.5	0.0317_5	41.4	42.1	−0.7	19
5	C(17):C(17)PC	1.5	0.0298_5	49.0	49.3	−0.3	15
6	C(18):C(18)PC	1.5	0.0282	53.3	55.7	−0.4	19
7	C(19):C(19)PC	1.5	0.0267	61.8	61.3	0.5	19
8	C(20):C(20)PC	1.5	0.0253	66.4	66.5	−0.1	19
9	C(21):C(21)PC	1.5	0.0241	71.1	71.1	0.0	19
10	C(22):C(22)PC	1.5	0.0230	74.8	75.3	−0.5	19
11	C(17):C(16)PC	2.5	0.0308	43.2	43.1	0.1	up
12	C(15):C(13)PC	3.5	0.0364	18.8	18.2	0.6	15
13	C(16):C(14)PC	3.5	0.0339	28.4	28.0	0.4	15
14	C(17):C(15)PC	3.5	0.0317_5	37.7	36.5	1.2	16
15	C(18):C(16)PC	3.5	0.0298_5	44.4	44.0	0.4	14
16	C(18):C(15)PC	4.5	0.0308	38.1	37.7	0.4	up
17	C(16):C(12)PC	5.5	0.0364	11.3	11.8	−0.5	15
18	C(17):C(13)PC	5.5	0.0339	21.2	22.0	−0.8	15
19	C(18):C(14)PC	5.5	0.0317_5	31.2	30.9	0.3	16
20	C(19):C(15)PC	5.5	0.0298_5	39.0	38.7	0.3	14
21	C(19):C(14)PC	6.5	0.0308	31.8	32.2	−0.4	up
22	C(20):C(14)PC	7.5	0.0298_5	33.2	33.5	−0.3	14
23	C(12):C(16)PC	2.5	0.0364	21.7	21.0	0.7	15
24	C(13):C(17)PC	2.5	0.0339	30.5	30.3	0.2	15
25	C(14):C(18)PC	2.5	0.0317_5	39.2	38.4	0.8	16
26	C(15):C(19)PC	2.5	0.0298_5	44.8	45.5	−0.7	14
27	C(22):C(26)PC	2.5	0.0210_5	77.8	78.6	−0.8	up
28	C(11):C(17)PC	4.5	0.0364	13.9	14.2	−0.3	15
29	C(12):C(18)PC	4.5	0.0339	23.5	23.9	−0.4	15
30	C(13):C(19)PC	4.5	0.0317_5	32.5	32.4	0.1	16
31	C(14):C(20)PC	4.5	0.0298_5	39.8	39.9	−0.1	14
32	C(15):C(21)PC	4.5	0.0282	46.1	46.5	−0.4	20
33	C(16):C(22)PC	4.5	0.0267	52.8	52.5	0.3	20
34	C(17):C(23)PC	4.5	0.0253	57.9	57.8	0.1	20
35	C(18):C(24)PC	4.5	0.0241	62.7	62.6	0.1	20
36	C(20):C(26)PC	4.5	0.0220	70.7	71.0	−0.3	20
37	C(13):C(20)PC	5.5	0.0308	33.1	33.4	−0.3	up
38	C(11):C(19)PC	6.5	0.0339	17.3	17.6	−0.3	15
39	C(12):C(20)PC	6.5	0.0317_5	25.6	26.5	−0.9	16
40	C(13):C(21)PC	6.5	0.0298_5	34.1	34.3	−0.2	14
41	C(16):C(24)PC	6.5	0.0253	53.2	53.1	0.1	up
42	C(18):C(26)PC	6.5	0.0230	63.9	62.7	1.2	8
43	C(14):C(24)PC	8.5	0.0267	43.3	42.5	0.8	up
44	C(16):C(26)PC	8.5	0.0241	53.3	53.6	−0.3	up

[a]Lipids from Nos. 1–22 are C(X):C(Y)PC with X ⩾ Y, and the calculated values of T_m^{cal} (column 6) are obtained from $T_m = 162.26 - 3651.71\ (1/N) - 88.42\ (\Delta C/N)$. Lipids from Nos. 23–44 are C(X):C(Y)PC with X < Y, and the calculated values of T_m^{cal} (column 6) are obtained from $T_m = 157.68 - 3525.44\ (1/N) - 93.28\ (\Delta C/N)$.

[b]Unpublished calorimetric results from this laboratory.

The general equation of Huang (8) also can be revised based on the available T_m values of 44 points, and the resulting equation is $T_m = 153.67 - 1.73\Delta C - 1498.03\ (1/CL) - 139.66\ (\Delta C/CL)$ with $\sigma = 0.9986$ and RMSE = 0.90878. Judging from the correlation coefficient and the RMSE, the accuracy of the revised equation is inferior in comparison with the two new equations presented in this paper. The general equation of Huang (8) also can be considered separately for C(X):C(Y)PC with longer or with shorter *sn*-1 acyl chain. The resulting equations are: $T_m = 154.86 + 1.75\Delta C - 1521.03\ (1/CL) - 136.97\ (\Delta C/CL)$ with $\sigma = 0.9989$ and RMSE = 0.81287 for C(X):C(Y)PC with longer *sn*-1 acyl chain (X ⩾ Y), and $T_m = 148.92 + 3.01\ \Delta C - 1406.26\ (1/CL) - 166.33\ (\Delta C/CL)$ with $\sigma = 0.9993$ and RMSE = 0.63108 for C(X):C(Y)PC with longer *sn*-2 acyl chain (X < Y). Again, the accuracy of predicting T_m values for these two equations can be compared with that of Equations 1 and 2 according to their σ and RMSE values. Clearly, Equations 1 and 2 are better in predicting the T_m values for C(X):C(Y)PC.

The fact that Equations 1 and 2 are simpler and better than the general equation of Huang (8) can be attributed to the different structural models employed in deriving the structure-property relationship. The basic structural model used in deriving Equation 1 or Equation 2 is a dimeric model (Fig. 1B), whereas the earlier equation of

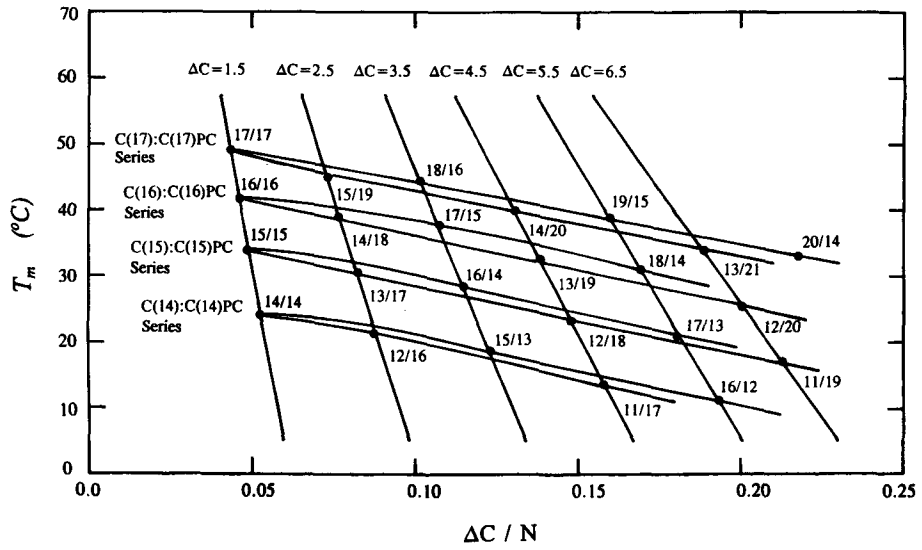

FIG. 3. The main phase transition temperature (T_m) *vs.* ΔC/N. The numeric ratio, X/Y, next to each data point is the ratio of the numbers of carbon atoms in the *sn*-1 and *sn*-2 acyl chains of the C(X):C(Y)PC molecular species, respectively. C(X):C(Y)PC as defined in Figure 1.

TOTAL CARBON ATOMS IN *sn*-1 ACYL CHAIN	TOTAL CARBON ATOMS IN *sn*-2 ACYL CHAIN																	
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
26										*0.2184* **59.0**	*0.1910* **63.3**	*0.1648* **67.4**	*0.1398* **71.4**	*0.1158* **75.1**	*0.0928* **78.8**	*0.0707* **82.2**	*0.0495* **85.6**	*0.0291* **88.8**
25									*0.2289* **54.0**	*0.2000* **58.7**	*0.1724* **63.1**	*0.1461* **67.3**	*0.1209* **71.3**	*0.0968* **75.2**	*0.0737* **78.9**	*0.0516* **82.4**	*0.0303* **85.8**	
24									*0.2099* **53.5**	*0.1807* **58.3**	*0.1529* **62.8**	*0.1264* **67.1**	*0.1011* **71.3**	*0.0769* **75.2**	*0.0538* **79.0**	*0.0316* **82.6**		
23								*0.2208* **47.9**	*0.1899* **53.0**	*0.1605* **57.9**	*0.1325* **62.5**	*0.1059* **67.0**	*0.0805* **71.2**	*0.0562* **75.2**	*0.0330* **79.1**			
22								*0.2000* **47.2**	*0.1688* **52.5**	*0.1392* **57.5**	*0.1111* **62.3**	*0.0843* **66.8**	*0.0588* **71.1**	*0.0345* **75.3**				*0.0526* **78.6**
21							*0.2113* **40.7**	*0.1781* **46.5**	*0.1467* **51.9**	*0.1169* **57.1**	*0.0886* **61.9**	*0.0617* **66.6**	*0.0361* **71.1**				*0.0550* **75.1**	*0.0753* **74.8**
20						*0.2239* **33.5**	*0.1884* **39.8**	*0.1549* **45.7**	*0.1233* **51.3**	*0.0933* **56.6**	*0.0649* **61.7**	*0.0380* **66.5**				*0.0575* **71.3**	*0.0787* **71.1**	*0.0989* **71.0**
19						*0.2000* **32.2**	*0.1642* **38.7**	*0.1304* **44.9**	*0.0986* **50.7**	*0.0685* **56.2**	*0.0400* **61.3**				*0.0602* **67.1**	*0.0824* **67.0**	*0.1035* **67.0**	*0.1236* **66.9**
18					*0.2131* **23.7**	*0.1746* **30.9**	*0.1385* **37.7**	*0.1045* **44.0**	*0.0725* **50.0**	*0.0423* **55.7**				*0.0633* **62.5**	*0.0864* **62.6**	*0.1084* **62.6**	*0.1294* **62.7**	*0.1494* **62.7**
17					*0.1864* **22.0**	*0.1475* **29.5**	*0.1111* **36.5**	*0.0769* **43.1**	*0.0448* **49.3**				*0.0667* **57.4**	*0.0909* **57.6**	*0.1139* **57.8**	*0.1358* **58.0**	*0.1566* **58.1**	*0.1765* **58.3**
16				*0.2000* **11.8**	*0.1579* **20.2**	*0.1186* **28.0**	*0.0820* **35.3**	*0.0476* **42.1**			*0.0435* **51.4**	*0.0704* **51.8**	*0.0959* **52.1**	*0.1200* **52.5**	*0.1429* **52.8**	*0.1646* **53.1**	*0.1852* **53.4**	*0.2048* **53.6**
15			*0.2157* **0.0**	*0.1698* **9.4**	*0.1273* **18.2**	*0.0877* **26.4**	*0.0509* **34.0**			*0.0462* **44.9**	*0.0746* **45.5**	*0.1015* **46.0**	*0.1268* **46.5**	*0.1507* **47.0**	*0.1733* **47.5**	*0.1948* **47.9**	*0.2152* **48.4**	
14		*0.2340* **-13.8**	*0.1837* **-3.0**	*0.1373* **6.9**	*0.0943* **16.1**	*0.0546* **24.6**			*0.0492* **37.5**	*0.0794* **38.4**	*0.1077* **39.2**	*0.1343* **39.9**	*0.1594* **40.6**	*0.1831* **41.3**	*0.2055* **41.9**	*0.2267* **42.5**		
13		*0.2000* **-17.7**	*0.1489* **-6.3**	*0.1020* **4.2**	*0.0588* **13.9**			*0.0526* **29.1**	*0.0848* **30.3**	*0.1148* **31.4**	*0.1429* **32.4**	*0.1692* **33.4**	*0.1940* **34.3**	*0.2174* **35.2**				
12		*0.1628* **-22.0**	*0.1111* **-9.9**	*0.0638* **1.2**			*0.0566* **19.4**	*0.0909* **21.0**	*0.1228* **22.5**	*0.1525* **23.9**	*0.1803* **25.3**	*0.2064* **26.5**						
11	*0.1795* **-40.9**	*0.1220* **-26.7**	*0.0698* **-13.8**			*0.0612* **8.1**	*0.0980* **10.3**	*0.1321* **12.3**	*0.1636* **14.2**	*0.1930* **16.0**	*0.2203* **17.6**							
10	*0.1351* **-47.1**	*0.0769* **-31.8**			*0.0667* **-5.2**	*0.1064* **-2.3**	*0.1429* **0.5**	*0.1765* **3.0**	*0.2076* **5.3**									
9	*0.0857* **-54.0**			*0.0732* **-21.1**	*0.1163* **-17.1**	*0.1556* **-13.5**	*0.1915* **-10.2**											

FIG. 4. Calculated T_m values for 173 molecular species of saturated diacyl phosphatidylcholines. The X and Y values in C(X):C(Y)PC are illustrated on the vertical and horizontal axes, respectively. The values of ΔC/N and T_m are shown in a square box which represents a defined molecular species of C(X):C(Y)PC. The ΔC/N and T_m values are printed in italic and boldface, respectively. Abbreviations as in Figure 3.

Huang (8) is based on a structural model of the monomer. Much structural information is inherently incorporated with a dimeric unit; consequently, a dimeric model ultimately leads to improved characterizations of the structure-property relationship for phospholipid bilayers.

Finally, we should comment on the physical meaning of the individual term in each of the two derived equations. The first term, a constant, corresponds to the maximal T_m value for the lipid bilayer comprised of phosphatidylcholine molecules with infinitely long acyl chains. The second term relates to the negative value of the inverse of the minimal hydrophobic thickness (N). As the minimal hydrophobic thickness becomes progressively larger, the T_m value correspondingly becomes higher. The third term can be regarded as a perturbation term. The close chain-chain interaction in the gel-state bilayer may be perturbed by the bulky chain terminal methyl groups and this perturbation is reflected by the negative value of ΔC (15,16). At a constant value of N, the ratio of $\Delta C/N$ in the third term is negative, thus making a negative contribution to the overall T_m value of the lipid system under study. Briefly, the equations derived in this paper emphasize that the T_m values of saturated diacyl phosphatidylcholines in excess water depend critically on the interplay of two structural parameters, ΔC and N, obtained from the dimeric lipid molecules in the gel-state bilayer.

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Phosphatidylcholine as Substrate for Human Pancreatic Phospholipase A_2. Importance of the Physical State of the Substrate

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The long-chain phosphatidylcholine/sodium cholate aqueous system as substrate for human pancreatic phospholipase A_2 (PLA_2) was investigated. At a constant phosphatidylcholine (PC) concentration of 8 mM, the enzyme activity increased with a decrease in cholate (C) concentration up to a PC/C ratio of approximately 0.8 and then rather abruptly decreased to lower values at a ratio above 1.5. At ratios between 0.8 and 1.5, an increasing lag phase in the PLA_2 activity was seen, indicating a progressive decrease in substrate availability to the enzyme. Reaction mixtures with a PC/C ratio of up to 0.67 were optically clear solutions composed of mixed bile salt/PC micelles of increasing mixed micellar aggregate size. Ratios between 0.67 and 1.5 were characterized by an increase in turbidity (at 330 and 450 nm) due to increasing formation of vesicles or liposomes. Above a PC/C ratio of 1.5, a sharp increase in turbidity was seen due to increasing formation of bilayer structures other than vesicles. Pure vesicles obtained by dialysis of mixed micellar solutions were not hydrolyzed by the enzyme. Addition of bile salts reversed the inhibition which was accompanied by a decrease in turbidity. Phosphatidylcholine was preferred as substrate for human PLA_2 when present in large mixed disc-like bile salt micelles. Vesicular or other types of lamellar liquid-crystalline phases of long-chain phosphatidylcholine did not serve as substrate for PLA_2.
Lipids 28, 371-375 (1993).

The activity of pancreatic phospholipase A_2 (PLA_2) [EC 3.1.1.4] against long-chain unsaturated phosphatidylcholine (PC) dispersed in bile salt (BS) solution depends on the molar PC/BS ratio (1). Optimal activity has been reported to occur at a ratio of about 0.5, varying somewhat with the structure of the components. PLA_2 was found not to hydrolyze PC at a PC/BS molar ratio greater than 1 or less than 0.2 (1). With respect to the physical state of the substrate, it was observed that an optically clear micellar solution was obtained at a PC/BS molar ratio below 0.4. The substrates used were human bile, intralipid emulsions or PC from egg yolk dispersed in a mixed trihydroxy-dihydroxy-conjugated bile salt solution; the physical state of the substrates was not further characterized. Hoffman *et al.* (2) studied the activity of porcine PLA_2 on egg PC/cholate (C) mixed micellar solutions at 5 mM PC. They found that the enzyme activity was related to the molar PC/C ratio within the mixed micelle rather than to the bulk concentration of PC. When the ratio of micellar PC to C within the micelle was varied from 0.2 to 2.0, the enzyme activity was increased 10-fold. Long-chain unsaturated PC, which forms bilayer structures (vesicles) in water, has generally been found to be a poor substrate for PLA_2 (3,4). PC/BS mixed micellar aggregates are thus good substrates for PLA_2 but it is unclear to what extent other types of PC aggregates will function as substrates. In view of the increase in knowledge that has been gained in recent years on the physical state of the aqueous long-chain PC/BS system (5-7), it seemed of interest to further characterize the interaction of PLA_2 with experimentally defined physical substrate structures. A study with a partially similar objective was published (8) when the present work was well under way.

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Abbreviations: BS, bile salt; C, cholate; IMC, intermicellar concentration; MW, molecular weight; PC, phosphatidylcholine; PLA_2, phospholipase A_2; SDS, sodium dodecylsulfate; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. PC, prepared from soybean (Epikuron 200), was kindly supplied by Lucas Meyer (Hamburg, Germany), it was better than 95% pure as confirmed by thin-layer chromatography (TLC). The main fatty acid components were: saturated fatty acids, 18%; 18:1, 12%; 18:2, 65%; and 18:3, 5%. Sodium cholate (C) was purchased from Serva (Heidelberg, Germany) and was better than 99% pure as judged by TLC. Human PLA_2 was prepared from pancreatic juice essentially according to Grataroli *et al.* (9). The enzyme was pure according to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis. A preparation with an optical density of 1.46 at 280 nm was used. Centricon-10 [10,000 molecular weight (MW) cutoff] and Centricon-100 (100,000 MW cutoff) filters were products of Amicon (Amicon Division, W.R. Grace and Co., Danvers, MA). The filters were used as described in the manufacturer's manual. The radioactive products 1-palmitoyl-2-[9,10-^{3}H]oleoyl PC and [^{14}C]cholate were from Amersham (Buckinghamshire, England).

Determination of PLA_2 activity. The availability of substrate for the enzyme was measured titrimetrically using a Mettler pH stat assembly (Mettler Instruments A.G., Zürich, Switzerland). Ten mL portions of the PC/C dispersions were pipetted into glass vials, the pH was adjusted to 8.0 and 10-20 μL of the enzyme solution was added. The pH was maintained at 8 for at least 10 min, and the activity was calculated from the slope of the titration curve in μmole/min. With samples with a PC/C ratio of 0.8 to 1.5, a latency period was initially seen before the steady state was reached. This type of titration curve is often observed with PLA_2, and initial rates as well as steady state rates have been calculated. By extrapolating the steady state curve to the time axis, the lag time (in min) was calculated.

Preparation of micellar solutions and vesicles. PC in aqueous C solution was dispersed as described by Almog *et al.* (6) either by hydration of lipid films in 135 mM NaCl or by rapid dilution of concentrated mixed micellar dispersions into 135 mM NaCl solutions. In the former case, PC and NaC were weighed into glass flasks and dissolved in chloroform/methanol (1:1, vol/vol). The solvent was

evaporated to form a thin film, and the flasks were kept under vacuum overnight to remove residual solvent. The lipids were then dispersed by adding the appropriate volume of buffer that was 135 mM in NaCl, 1 mM in Ca^{2+} and 0.5 mM in Tris/HCl, pH 8.0. Equilibration was rapid and the dispersions were used 1–2 h after preparation.

In the dilution experiments, aqueous solutions were prepared which were 100 mM in PC and 50–100 mM in NaC. These were clear micellar solutions, except for the PC/C solutions of ratios 100:62.5 and 100:50, which were turbid. Dilutions were then made by mixing 8 mL of the above solutions with 92 mL of the above buffers, or buffers with NaC when appropriate, under constant stirring. This way, dilutions of PC were obtained in the PC/C range from 8:32 to 8:4, which all contained a dilution of PC of 1:12.5. The rate of vesiculation was fast and maximum turbidity, when present, was reached after 1–2 h; this was followed by a steady state over 24 h. The solutions were in general allowed to "age" at room temperature overnight before being used as substrates. Turbidity of the dispersions was measured at 330 and 450 nm using a Hitachi U-1 100 spectrophotometer (Tokyo, Japan).

Filtration of PC dispersions using Centricon filters. Generally clear filtrates were obtained. The filtration rates were slow in some cases with dispersions of high turbidity. In such cases better filtration rates were obtained with the Centricon-10 filters if the dispersion were pre-filtered through a Centricon-100 filter. In the filtration studies, radioactively-labeled PC and C were used to calculate concentrations in the filtrates. Appropriate amounts of PC and C were mixed in a glass flask with 1-palmitoyl-2-[9,10-^{3}H]oleoyl PC and [^{14}C]cholate in chloroform/methanol solution. The solvent was evaporated after the solutions became clear. The dried films were dispersed in buffer under constant stirring. Samples were taken for determination of radioactivity before filtration and from the filtrates.

RESULTS

The importance of the PC/C ratio for the activity of PLA_2. The PC concentration in the bulk phase was kept constant at 8 mM and the C concentration was varied from 4 to 32 mM. In the experiments, the results of which are given in Figure 1, the dispersions were prepared by hydrating the dry PC/C film in buffer. The films were dispersed by the use of a magnetic stirrer. At low PC/C ratio the solutions were clear and the optical density at 330 and 450 nm low until a PC/C ratio of 0.67. At this point the values started to increase more sharply at 330 than at 450 nm, with a discontinuity at a molar ratio of PC/C of 1.5. Increasing turbidity of the solutions above a ratio of 0.67 was also observable in normal light. The activity of PLA_2 increased from the PC/C of 0.25 up to approximately 0.8 and then dropped abruptly. At PC/C ratios of 0.89 to 1.14, a lag phase was seen before a constant rate of hydrolysis was attained. The lag time increased with increasing PC/C ratio and became very long; for a PC/C ratio of 1.6 a constant and low rate of hydrolysis could only be seen after 90 min of incubation. At even higher ratios the lag time became too long to be experimentally measured.

Vesicles (or liposomes) as substrate for PLA_2 and the effect of adding C. Liposomes were prepared by diluting

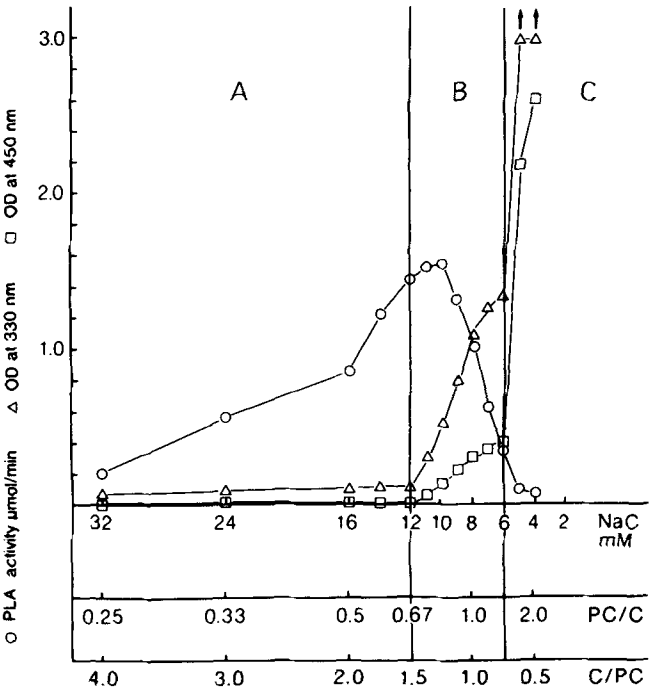

FIG. 1. Plots of phospholipase A_2 (PLA_2) activity (○), optical density (OD) at 330 nm (△) and 450 nm (□) over concentration of cholate (C), the phosphatidylcholine (PC)/C ratio, and C/PC ratio. The dispersions were 8 mM in soybean PC, 135 mM in NaCl and 1 mM in CaCl. NaC, sodium cholate.

a micellar solution of PC/C, 100 mM of each, 12.5 times in 135 mM NaCl and by leaving it overnight. This dispersion had an optical density at 450 nm of 0.33 and was dialyzed against two changes of 10 times the volume of the sodium chloride buffer. During the dialysis the optical density increased to 0.49. The liposome suspension was not hydrolyzed by PLA_2. When C was added back to the liposomal dispersions, they cleared and became substrates for the enzyme. Such an experiment is seen in Figure 2. Turbidity of the liposomal dispersions decreased after adding C. PLA_2 activity was measured 2 h after the addition of C. At low cholate and high turbidity a lag time was seen; at a concentration of 3.8 mM (PC/C 2.0) the lag time was 47 min, at 7.4 mM C (PC/C 1.0) no lag time was seen.

After diluting PC/C (100:100) 12.5 times in 135 mM NaCl, maximal turbidity was reached after 1–2 h, followed by a steady state for at least 24 h. The PLA_2 activity followed the same pattern with a tendency to a slow decrease over time (not shown). The results indicate that formation of liposomes (vesiculation) is a fast process and that the liposomes are rather stable. They also show that dissolution of vesicles by bile salt is a fast process.

Filtration of PC/C dispersions of different ratios through Centricon filters. The results of these experiments are seen in Figures 3 and 4. No PC passed the Centricon-10 filter with a molecular cutoff of MW 10,000 at any BS concentration (Fig. 3). This means that no mixed micelles passed this filter. The C concentration in the filtrates increased linearly with total C concentration, and thus represents the intermicellar concentration of C (monomers + simple C micelles) (10). From these figures the PC/C ratio in aggregated form in the bulk phase can be calculated and

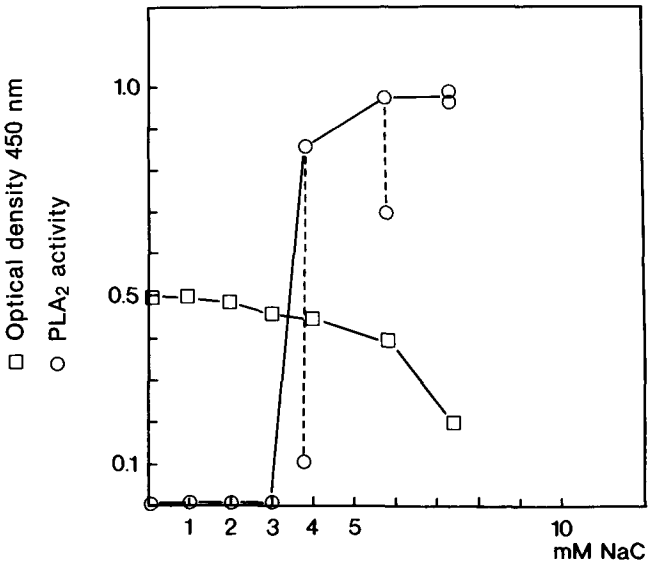

FIG. 2. Phospholipase A_2 (PLA_2) activity (μmol/min) (○) and optical density at 450 nm (□) of liposomal dispersions of 8 mM PC 2 h after the addition of sodium cholate (NaC) to obtain different final concentrations of cholate. At intermediate bile salt concentrations there was a lag phase before PLA_2 activity reached a steady state. In the figure the initial rates are connected by a broken line with the corresponding final steady state rates. At low cholate concentrations, no PLA_2 activity was seen.

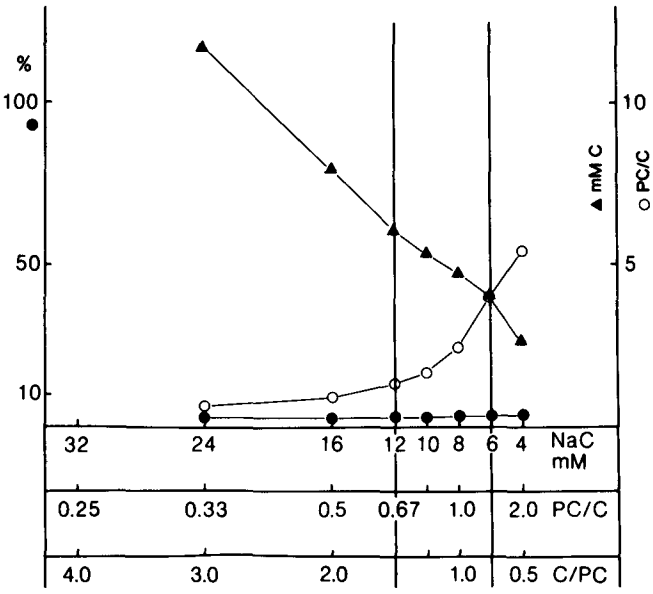

FIG. 3. Results obtained in the Centricon-10 filtration experiments of dispersions of 8 mM phosphatidylcholine (PC) in cholate (C) solutions at different PC/C ratios. The symbols indicate: percent of PC in the filtrate (●); concentration of cholate (mM) in the filtrate (▲) which equals the intermicellar concentration (monomers + simple micelles), PC/C ratio in the aggregated form at different cholate concentrations (○). NaC, sodium cholate.

is given in Figure 3. It increases from 0.64 at a PC/C ratio of 0.33 in the bulk phase to 5.49 at a PC/C ratio of 2.

When filtered through Centricon-100 filters, the filtrates were, in principle, clear (some filters were leaking and the

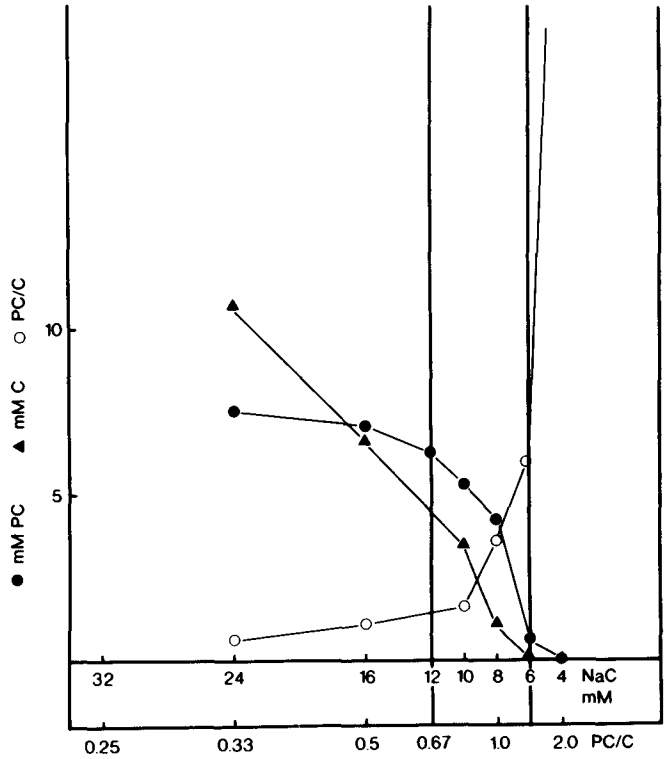

FIG. 4. Results obtained in the Centricon-100 filtration experiments of dispersions of 8 mM phosphatidylcholine (PC) in cholate (C) solutions at different PC/C ratios. The symbols indicate concentrations of PC (mM) in the filtrates out of a total of 8 mM (●); concentration of C in aggregate form in the filtrates (mM) (△). PC/C ratio in aggregated form at different PC/C ratios in the bulk dispersion (○). NaC, sodium cholate.

filtrates were discarded). Figure 4 shows the concentration of PC in mM (total PC 8 mM) in the filtrates as a function of the PC/C ratio. PC passed through the filters and therefore was in mixed micellar solution. With increasing PC/C ratio, the concentration of PC in the filtrates decreased steeply after a PC/C ratio of 0.67, and PC was absent from the filtrate at a ratio of 2. This indicates that with a decrease in C, vesicles and other aggregated forms of PC were formed that were not able to pass through the Centricon-100 filter. The concentration of C in the filtrate fell sharply with decreasing total concentration of C. The C found in the filtrates was in intermicellar and mixed micellar form. As the intermicellar concentration of C was obtained from the Centricon-10 experiments, these figures together can be used to calculate the amount of C in aggregated form in the Centricon-100 experiments, as well as the PC/C ratios. The latter figures from the Centricon-10 and Centricon-100 experiments were similar.

DISCUSSION

The physiological substrate for pancreatic PLA_2 in the small intestinal content is long-chain PC in BS dispersions. Although such systems have been studied *in vitro* (1,2) as substrate for PLA_2, no systematic determination of the enzyme activity in relation to the physical state of the substrate had been done until recently (8). In the present work, the studies were done at a constant PC concentration of 8 mM and at varying concentrations of sodium

C. The solutions were used as substrate for human pancreatic PLA_2, and the enzyme activity measured was correlated with the PC/C ratio. The physical state of the dispersions was defined by measuring the optical density at 330 and 450 nm and by filtering through synthetic filters with defined MW cutoffs. The interpretation of the data was aided based on studies on aqueous BS/lecithin systems that have been published in recent years. Mazer *et al.* (5) have shown that at low PC/C ratios, simple BS micelles coexist with small-size mixed BS lecithin micelles. As the PC/C ratio increases, the number of simple micelles decreases to furnish BS for the formation of mixed micelles. The solubilization of further PC at an increased PC/C ratio leads to a reorganization of mixed micellar size and structure. The data of Mazer *et al.* (5) seem to indicate that disk-shaped mixed micelles are formed which grow in size up to the mixed micellar phase limit. Above this limit bilayer structures in the form of unilamellar vesicles or liposomes appear; these are metastable and precipitate as bilayered structures other than vesicles. Almog *et al.* (6) have reported that for equal total concentration of PC and C, any mixture with a concentration of 8 mM or less was vesicular. The size of the vesicles was dependent on the dilution, and for the 12.5 times dilution used here the hydrodynamic radius was around 500Å. The turbidity at a given dilution thus depends on the vesicular size and the number of vesicles. If the results of our experiments are interpreted against the background discussed above, it appears that in Figure 1, the mixed micellar phase boundary can be located by the appearance of turbidity that occurs at a PC/C ratio of 0.67. The turbidity is due to the formation of vesicles. The abrupt increase in the turbidity at a PC/C ratio of 1.5 represents a transition of vesicles to precipitated bilayer structures (5). Figure 1 thus can be divided into three zones marked A, B and C. Zone A contains monomeric C in equilibrium with simple micelles and mixed PC/C micelles. The coexistence boundary, defined by Mazer *et al.* (5), separates regions in which both simple and mixed micelles exist from the region in which only mixed micelles are present, and is indicated by an increase in the hydrodynamic radii but cannot be located in our experiments. In zone A, PLA_2 activity increases linearly up to a PC/C ratio of 0.5 and thereafter shows a steeper increase in activity into Zone B, where vesicles start to form. In our experiments the total concentration of PC is constant at 8 mM; hence increase in PLA_2 activity must be due to increased availability of PC to the enzyme. Therefore, it appears that the large-size, mixed disk-like micelles are the preferred substrate for PLA_2. Zone B contains large mixed PC/C micelles coexisting with an increasing number of unilamellar vesicles, which is reflected in a sharp decrease in enzyme activity. When the relative C concentration is too low, the vesicles aggregate to form precipitated bilayer structures which are essentially unavailable as substrate. This transition starts at a PC/C ratio of 1.5 and is indicated by a sharp increase in turbidity.

Inside Zone B, starting at a PC/C ratio of 0.89, a lag phase in the PLA_2 activity begins to appear, which increases to 90 min at a ratio of 1.6. The lag phase, which increases with the PC/C ratio, can likely be explained by decreasing availability of PC in the vesicular structures to the enzyme. The fact that the activity starts after a lag phase is probably due to product activation, as fatty acids and lysoPC affect the bilayer structure (10,11). By removing C from vesicles by dialysis, pure metastable vesicles are obtained (6). Such vesicles are not a substrate for PLA_2. Adding C back to the system resulted in a decrease in the turbidity and appearance of enzyme activity after the addition of PLA_2 (see Fig. 2). At low cholate concentration, a long lag phase occurred. The presence of cholate in the bilayer is therefore important for the activity of PLA_2. The studies with Centricon filters allowed the calculation of the PC/C ratio in the aggregated form and showed an increase from 1.2 to 4 in Zone B. For optimal activity, the PC/C ratio in the aggregates should be around 2. It has previously been shown that in bilayer structures of PC within a limited temperature region just above the transition temperature (in the absence of bile salts), the PLA_2 activity increases at a gradually increasing rate due to a change in the transition temperature because of the appearance of products of the enzyme reaction (3,4). Structural defects in the substrate bilayer introduced by sonication have also been shown to facilitate the hydrolysis by PLA_2, and this has led to the conclusion "that structural irregularities in the packing of the substrate molecules facilitate the action of PLA_2 on bilayers" (4). In an analogous way it appears that such irregularities can be induced by BS, thus affecting the activity of PLA_2. The presence of low PC/BS molar ratios (0.17–0.28) in native human bile (1) explains why this substrate is almost unavailable to PLA_2. Figures obtained on human duodenal contents, however, indicate PC/BS molar ratios of around 0.6, which should be optimal for attack by human PLA_2 (12).

The results of the present study are, in many respects, confirmatory of those of a previous report (8) which was published when the present study was essentially finished. In the work by Gheriani-Gruszka *et al.* (8), the hydrolysis of egg PC in mixtures with C by porcine pancreatic PLA_2 was studied. It was found that increasing the C/PC ratio (decreasing the PC/C ratio) in the micelle resulted in a decreased (increased) initial velocity of hydrolysis. Special attention was given to the hydrolysis of C containing unilamellar vesicles. These were found sensitive to the ratio of C/PC in the vesicles, and it was concluded that large structural fluctuations are introduced into the vesicles by the presence of C, thus promoting the activation of the enzyme. In the present study, C was added back to the vesicles obtained by dialysis, and the results were also interpreted to indicate that the presence of C in the bilayer is important for the activity of PLA_2. Another interpretation of the results would be that adding C to the vesicles results in the formation of mixed micelles which are substrates for the enzyme.

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Purification and Properties of an Extracellular Lipase from *Pythium ultimum*[1]

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An extracellular triacylglycerol lipase (EC 3.1.1.3) from *Pythium ultimum* strain No. 144 was purified by ammonium sulfate precipitation, and by diethylaminoethyl Sepharose CL-6B and Sephacryl S-200 chromatography. The purified enzyme preparation showed a prominent polypeptide band in polyacrylamide gel electrophoresis, associated with esterase activity according to activity staining. Molecular weight of the protein was estimated at 270 kD using gel filtration on Sephacryl S-200, and 68 kD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicating that the enzyme may be a tetramer. The optimum pH and temperature for activity of the enzyme were 8.0 and 30°C, respectively. Activity was reduced by Co^{2+}, Fe^{2+}, Sn^{2+} and Mn^{2+} and stimulated by Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} and surfactants such as taurocholic acid, Triton X-100, *n*-octyl glucoside, *n*-dodecyl-β-D-maltoside, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate(CHAPS), and 3-[-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate. The apparent maximum specific activity was 42 μmole/min/mg in the absence of CHAPS and 77 μmole/min/mg in its presence. The reaction rate was progressively higher with increasing number of double bonds in the substrate, and the enzyme showed a preference for triacylglycerols containing fatty acids having the *cis* double bond configuration.
***Lipids* 28, 377–382 (1993).**

Lipases (EC 3.1.1.3, glycerol ester hydrolases) mainly catalyze the hydrolysis of triacylglycerols to diacylglycerols and free fatty acids, but some lipases are also known to catalyze the hydrolysis of mono- and diacylglycerols, and the interesterification of oils (1,2). Lipases are produced by a wide variety of organisms, including fungi (3). Lipases from several fungi representing mostly the *Ascomycotina*, *Deuteromycotina* and *Zygomycotina* have been isolated and partially characterized. Microbial lipases have become industrially important in the modification of fats and oils (4).

Extracellular lipase production by the filamentous fungus *Pythium ultimum* strain No. 144, which belongs to the primitive fungal class Oomycetes, was suggested in our previous studies on the production of fungal oil enriched in arachidonic and eicosapentaenoic acids; *i.e.*, cultures readily utilized soybean oil as a co-carbon substrate for growth (5,6). This report describes the isolation, purification and properties of an extracellular lipase from this fungus.

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Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO,3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DEAE, diethylaminoethyl; DG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; FFA, free fatty acids; MG, monoacylglycerol; SDS—PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TG, triacylglycerol; TLC, thin-layer chromatography; V_{max}, maximum specific activity.

MATERIALS AND METHODS

Materials. Trizma maleate (Tris maleate), lipase substrate (50%, vol/vol olive oil emulsion), tristearin, triolein, trilinolein, trilinolenin, trielaidin, taurocholic acid, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), Fast blue RR salt, α-naphthyl acetate, bovine serum albumin (BSA), ovalbumin, carbonic anhydrase, α-lactalbumin, diethylaminoethyl (DEAE) Sepharose CL-6B, Sephacryl S-200 and reagents for polyacrylamide gel electrophoresis were from the Sigma Chemical Co. (St. Louis, MO). Dye reagent for protein assays was purchased from Bio-Rad Laboratories (Richmond, CA), and molecular weight standards for gel chromatography were obtained from Boehringer Mannheim (Mannheim, Germany). All other reagents were of the highest purity available.

Fungus. Pythium ultimum strain No. 144, obtained from D.J.S. Barr of the Biosystematic Research Center, Central Experimental Farm (Ottawa, Canada), was maintained in Vogel's medium (5) at 18°C with rotary shaking at 120 rpm. The fungus was transferred to new medium at approximately 6-d intervals.

Lipase production. The fungus was cultivated for 6 d at 18°C in 250-mL Erlenmeyer flasks containing 100 mL Vogel's medium and 2 mL soybean oil in a Lab-line rotary incubator shaker at 120 rpm. Inoculations were made with 5 mL of blended mycelium from a 6-day-old culture grown as described above. Mycelia were separated from the medium by suction filtration in a Buchner funnel, and the lipases were then isolated from the medium.

Lipase purification. Proteins were precipitated from the culture medium with ammonium sulfate (85% saturation). After standing in the ammonium sulfate solution for 16 h at 4°C, the precipitate was collected by centrifugation at 15,000 rpm for 30 min and then dissolved in 50 mM sodium phosphate buffer pH 7.5 containing 10% glycerol (buffer A). Undissolved materials were removed by centrifugation as before, and the solution was dialyzed against the same buffer overnight. The dialyzate was placed on a DEAE-Sepharose CL-6B column (25 mm × 100 mm) which had been equilibrated with Buffer A. The column was washed sequentially with 100 mL each of Buffer A containing 0.05, 0.1, 0.2, 0.3 and 0.5 M NaCl and finally with 50 mM sodium phosphate buffer pH 7.5 (Buffer B) containing 1.0 M NaCl. The highest lipase activity was found in the fraction eluted with Buffer B containing 1.0 M NaCl. This fraction was dialyzed against Buffer A and concentrated by passage through a DEAE-Sepharose CL-6B column (10 mm × 20 mm). Lipase was eluted with a minimal volume of Buffer B containing 1.0 M NaCl, and the eluate was dialyzed against Buffer A and then placed onto a Sephacryl S-200 column (15 mm × 400 mm) previously equilibrated with Buffer A. The active fractions were concentrated by DEAE column chromatography as before. Glycerol (10%) was added to the buffer during purification and long term storage (4–6 mon) to

prevent the loss of enzyme activity. All procedures were carried out at 0–4°C.

Assay of lipase activity. Lipase activity was measured by the method of Ota *et al.* (7). Composition of the reaction mixture was 2 mL 0.2 M Tris maleate-NaOH buffer pH 8.0, 1 mL 0.06 M $CaCl_2$, 5 mL deionized water, 1 mL olive oil emulsion (50% vol/vol olive oil) and 1 mL of the enzyme preparation appropriately diluted with Buffer A. When substrates other than olive oil were used, they were emulsified in 5 mL deionized water with or without CHAPS (1.6 mM), but otherwise the reaction mixture was the same as described above. The emulsion was made using a Ross micro-mixture emulsifier (Charles Ross & Son Co., New York, NY). The reaction mixture was incubated at 30°C with constant reciprocal shaking (45 cycles/min). After 60 min, 20 mL of an acetone/ethanol (1:1, vol/vol) solution were added to stop the reaction, and the free fatty acid content was determined by titration with 0.05N aqueous NaOH. The reaction mixture without enzyme (blank) was also incubated as described above, but the enzyme was added prior to titration. The amount of product formed during incubation was calculated by subtracting the amount of free fatty acid in the blank from that of the reaction mixture after incubation with the enzyme. One unit of lipase was defined as the amount of enzyme required to liberate 1 μmol of fatty acid per min under the conditions specified. The assay method for the *P. ultimum* lipase was standardized using the olive oil emulsion as the source of substrate. The time course of hydrolysis was measured at various enzyme dilutions at constant substrate concentrations to establish the incubation time for the assay. The reaction was linear over time for 60–120 min at enzyme/substrate ratios where fatty acid release was between 35 to 70 μmol. Thus, 60 min was selected as the incubation time.

Values for the apparent K_m and V_{max} (maximum specific activity) were determined from reciprocal plots of substrate (triolein) concentrations *vs.* initial reaction rates (8). Initial reaction rates for all substrate concentrations were determined by appropriately diluting the enzyme preparation. Optimum temperature for the reaction was determined by measuring activity after incubation for 60 min at temperatures between 20 to 60°C. Tris maleate/NaOH (0.2 M) buffer at different pH values (4 to 10) was used to determine pH dependence of the enzyme at 30°C.

Treatments within each experiment were done in triplicate, and each experiment was done at least twice.

Positional specificity determination. The course of triolein (0.1 mL triolein in 10 mL reaction mixture as described above) hydrolysis by the lipase was followed by thin-layer chromatography (TLC). Small portions of the reaction mixture were removed at 0, 1, 3 and 5 h of incubation period. Diethyl ether was added to stop the reaction and to extract the products. The extraction was done at 4°C, and the extract was kept at 4°C prior to analysis. Samples were applied to silica gel layers (250 μm thick) on 20 cm × 20 cm glass plates, and the plates were developed in chloroform/acetone (96:4, vol/vol) as the solvent system. Components of the reaction mixture were visualized with iodine vapor and identified by comparison of their migration rates with those of known standards (Sigma).

Effects of temperature, metal ions, chelators and surfactants on lipase activity. The effect of temperature on lipase activity was determined by measuring the activity remaining after exposing the enzyme for 60 min to various temperatures (20–60°C), after which time the enzyme activity was determined under the assay condition described above.

To determine the effects of metal ions and various surfactants and chelators on lipase activity, the enzyme was incubated for 10 min at 20°C, in 0.2 M Tris-maleate-NaOH buffer, pH 8.0, containing the agents to be tested, and then enzyme activity was determined as before.

Molecular weight determination. Molecular weight of the purified lipase was determined by SDS-PAGE using 7.5% slab gels (9) and by gel filtration on a Sephacryl S-200 column (15 mm × 400 mm). In the case of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), BSA, ovalbumin, carbonic anhydrase and α-lactalbumin were used as standards. Proteins were stained on the gels with Coomassie blue. For gel chromatography, the column was calibrated using the Boehringer kit for molecular weight determination.

Activity staining. Native PAGE was performed on a 7.5% slab gel, and the enzymatically active (esterase) zone was identified by staining with 0.03% Fast blue RR salt, 0.05% α-naphthyl acetate and 1% acetone in 25 mL Tris-HCL at pH 7.4 (10).

Protein determination. Protein was determined colorimetrically at 595 nm using the Bio-Rad protein reagent according to the Bradford method with BSA as the standard.

RESULTS AND DISCUSSION

Enzyme purification. Most of the proteins in the medium were precipitated by ammonium sulfate. In the case of DEAE-chromatography, most of the proteins having essentially no lipase activity were removed from the column with a stepwise gradient of NaCl solution up to 0.5 M. Most of the lipase activity was eluted as a single peak with 1.0 M NaCl; the specific activity of the enzyme was increased 3.5-fold by this chromatographic step (Table 1). The specific activity was increased only slightly with the final purification step using Sephacryl S-200 gel chromatography. Total recovery of lipase activity was 22%. The active fraction was relatively pure according to native PAGE and activity staining (Fig. 1). However, minor proteins with relatively low molecular weights were also observed when the active fraction was subjected to SDS-PAGE (Fig. 2). Further purification was not achieved using DEAE-Cibacron blue 3GA-agarose or CM cellulose chromatography, both of which resulted in low recovery (less than 5%). The final specific activity of the lipase, after DEAE and Sephacryl chromatography, was about 63 units/mg protein which is relatively low compared to some of the fungal extracellular lipases reported previously, *e.g.*, 447 U/mg protein for *Goetrichum candidum* (11), 331 for *Aspergillus niger* (12), 3485 for *Mucor miehei* Lipase A (13) and 7638 for *Rhizopus delemar* (14). Clearly the activities of lipases from different fungal sources vary considerably. We have observed that the addition of surfactants to the incubation mixture increased activity of the *P. ultimum* lipase up to 161 units/mg protein (see below). Further studies have indicated that the amount and type of surfactant plays an important role in the activity of the *Pythium* lipase and that the activity could be increased

TABLE 1

Purification of an Extracellular Lipase from *Pythium ultimum* strain No. 144[a]

Steps	Volume (mL)	Protein (mg)	Specific activity[b] (units/mg protein)	Yield (%)
Media	1000	134	17.4	100
$(NH_4)_2SO_4$ precipitation	100	91	16.8	66
DEAE Sepharose CL-6B	10	15	60.5	39
Sephacryl S-200	5	8	62.9	22

[a]Details for each purification step are described in Materials and Methods. DEAE, diethylaminoethyl.
[b]Specific activity is defined as the amount of protein (mg) that catalyzed the release of 1 μmol of free fatty acid per minute from emulsified olive oil at 30°C and pH 8.0.

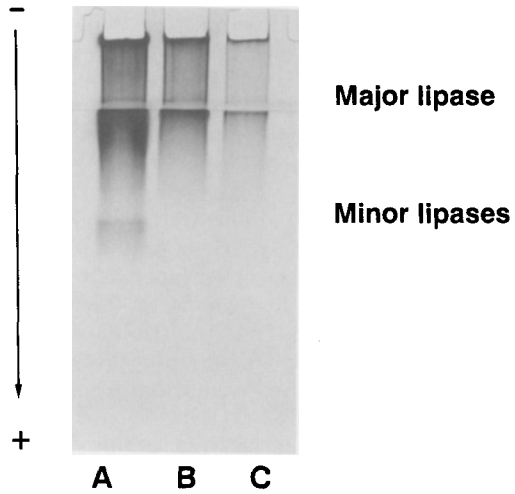

FIG. 1. Polyacrylamide gel electrophoresis (native) of crude and purified extracellular lipase preparations from *Pythium ultimum* with staining for esterase activity. A) Crude preparation; B) after diethylaminoethyl (DEAE) Sepharose CL-6B; C) after DEAE and Sephacryl S-200. The detailed method for activity staining is described in the Materials and Methods section.

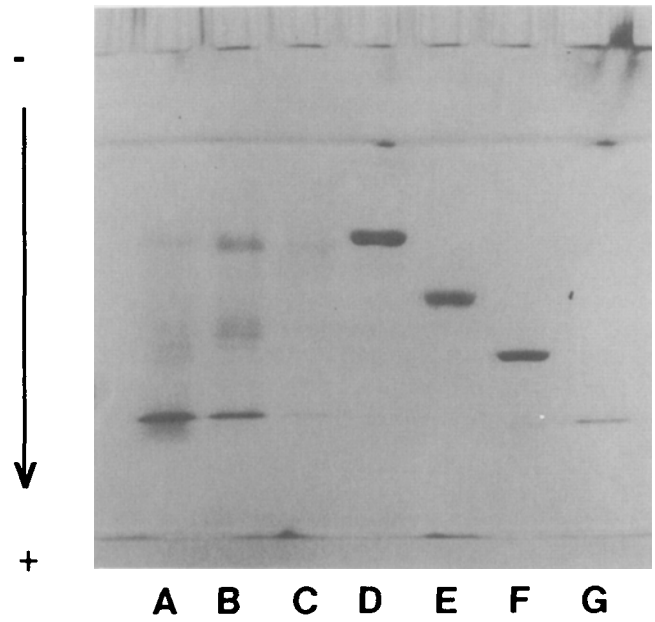

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified lipase from *Pythium ultimum*. A) Crude preparation; B) after $(NH_4)_2SO_4$ precipitation; C) purified lipase; D) bovine serum albumin (68 kD); E) ovalbumin (45 kD); F) carbonic anhydrase (29 kD); G) α-lactalbumin (14.2. kD).

tenfold over that of the control without surfactant (Mozaffar, Z., and J.D. Weete).

The enzyme preparation contained a prominent polypeptide possessing esterase activity according to activity staining on PAGE (Fig. 1), but there were also several minor polypeptides on the gel showing activity (Fig. 1A). The production of multiple lipases seems to be typical of fungi such as *A. niger* (12), *Saccharomycopsis lipolytica* (7), *G. candidum* NRRL Y-553 (15) and *Penicillium cyclopium* (16). It is not known whether the different *P. ultimum* lipases are the result of post-synthesis modifications of single gene products, such as proteolytic breakdown as suggested for lipases from *S. lipolytica* (7), of *N*-glycosylation as for the lipases from *G. candidum* (15) or of more than one lipase gene as suggested for the lipases from *P. cyclopium* (16). The prominent lipase from *P. ultimum*, which had the slowest mobility in the gel, was separated from the others by ammonium sulfate precipitation and DEAE chromatography, and it is this lipase that was further characterized.

Molecular weight. The estimated molecular weight of the major polypeptide in the purified extracellular lipase preparation from *P. ultimum* was 68 kD according to SDS-PAGE (Fig. 2). However, chromatography under nondenaturing conditions indicated that the molecular weight of the lipase was 270 kD, suggesting that the enzyme exists as a tetramer. Most fungal extracellular lipases are monomers having molecular weights ranging from 19 to 64 kD. Exceptions are a 207 kD lipase from *Candida deformans* (17) and that from *Neurospora crassa* which exists as a dimer with 27 kD molecular weight subunits (18).

Effect of temperature and pH. Hydrolytic activity was detected between 20 and 60°C, but optimum activity was at 30°C. The enzyme retained full activity when exposed to 20–40°C for 60 min prior to assay. However, activity decreased substantially when exposed to temperatures above 40°C and disappeared completely on treatment for 60 min at 60°C. This appears to be typical of most fungal lipases except for those from *R. delemar* (19) and *Humicola lanuginosa* No. 3 which exhibited activity at 60°C (20).

Activity of the *P. ultimum* lipase was observed over the range pH 4–10, but the pH optimum was 8. Most extracellular fungal lipases have acid or neutral pH optima, and very few are alkaline lipases.

Effects of metal ions, chelators and surfactants on lipase activity. The effects of various metal ions, chelators and surface active reagents on the activity of the extracellular lipase from *P. ultimum* were also investigated. Na^+, K^+, Ca^{2+} and Mg^{2+} enhanced activity by 56 to 150%, with the monovalent cations giving the strongest stimulatory activity (Table 2). Fe^{2+}, Co^{2+}, Sn^{2+} and Mn^{2+} reduced activity by 14 to 86% (Table 2). Perhaps the reduction in activity by the latter cations was due to their catalytic effects on oxidation, *i.e.*, their presence promoted the oxidation of unsaturated fatty acids. Similar results have been reported by others for lipases from different sources (17,20).

Six surfactants were tested for their effects on lipase activity by adding them to the reaction mixture containing the proprietary emulsified olive oil (Sigma) as the substrate. Whereas the surfactants were added to the reaction mixtures at concentrations below their critical micelle concentrations, some of their concentrations differed such that it was not possible to make direct comparisons of their effects on lipase activity in all cases. However, the surfactants generally stimulated lipase activity by 117 to 300% (Table 2). Triton X-100, CHAPS and CHAPSO were all added to the reaction mixture at a final concentration of 0.8 mM and exhibited the greatest stimulation of activity at 317, 367 and 400%, respectively (Table 2). Whether the stimulation of lipase activity was due to a direct interaction of the surfactant with the enzyme or to an alteration of emulsion properties which in turn affected activity is not known. However, different surfactants affect fungal lipases differently. For example, Triton X-100 and *n*-octyl glycopyranoside inhibited lipases from *Candida rugosa* and *G. candidum*, but Triton X-100 stimulated the lipase from *Rhizopus arrhizus* (21).

Kinetic parameters. Apparent K_m and V_{max} values obtained for lipase with triolein as the substrate in the absence of CHAPS were 25 mM and 42 μmol/min/mg protein, respectively. These values were 20 mM (K_m) and 77 μmol/min/mg protein (V_{max}), respectively, when CHAPS was added to the reaction mixture. The apparent K_m values with and without CHAPS were not significantly different, but the apparent V_{max} values for the reaction in the presence of the surfactant was significantly higher than without it ($P < 0.001$). The increased V_{max} is probably due to an improvement in emulsion properties of the reaction mixture which favor more enzyme molecules becoming active. Only up to 30 mM triolein could be emulsified in the absence of CHAPS. Above this concentration, dispersibility of the oil in aqueous solutions decreased, and a thick cream was formed. On the other hand, 60 mM triolein was readily emulsified in the presence of CHAPS.

Activity as a function of substrate unsaturation. The lipase from *P. ultimum* has marked preference for substrates containing *cis*-unsaturated fatty acids (Table 3). For example, the rate of hydrolysis of trielaidin (18:1 *trans*-9) was 82% less than that for triolein (18:1 *cis*-9). There was no detectable hydrolysis of tristearin by this enzyme. Since tristearin and trielaidin are solid at room temperature, the poor lipase activity toward these substrates may have been due to poor emulsification, although an emulsifier was used to disperse the substrates in water.

TABLE 2

Effects of Cations, Chelators and Surface Active Agents on the Activity of the Extracellular Lipase from *Pythium ultimum* strain No. 144

Substances[a]	Activity[b] (μmol/min/mg protein)	Relative activities[c]
Control (none)	40.4 ± 0.8[d]	100
Sodium chloride (500 mM)	101.0 ± 3.2[f]	250
Potassium chloride (500 mM)	101.4 ± 4.1[f]	250
Calcium chloride (6 mM)	63.0 ± 2.0[f]	156
Magnesium chloride (5 mM)	75.1 ± 2.4[f]	186
Ferrous chloride (5 mM)	17.4 ± 0.8[f]	43
Manganous chloride (5 mM)	34.7 ± 1.2[e]	86
Cobaltous chloride (5 mM)	28.7 ± 0.8[f]	71
Stannous chloride (5 mM)	5.7 ± 0.8[f]	14
EDTA (5 mM)	32.7 ± 1.2[e]	81
EGTA (5 mM)	36.0 ± 1.2[e]	89
Taurocholic acid (4 mM)	107.9 ± 4.0[f]	267
Triton X-100 (0.8 mM)	128.1 ± 4.0[f]	317
n-Octyl glucoside (2.5 mM)	80.8 ± 3.2[f]	200
n-Dodecyl-β-D-maltoside (0.02 mM)	87.7 ± 3.2[f]	217
CHAPS (0.8 mM)	148.3 ± 4.4[f]	367
CHAPSO (0.8 mM)	161.6 ± 4.8[f]	400

[a]EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*-(β-aminoethyl-ether)*N,N,N',N'*-tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHAPSO, 3-[C3-cholomidopropyl)dimethylamonio]-2-hydroxy-1-propanesulfonate. Concentrations of the additives in the reaction mixture during activity determination are shown in parentheses.

[b]Each value is the mean ± SD of three determinations. Statistical significance was assessed by Student's *t*-test. The significance of differences between respective values is shown as *P*; $P < 0.01$ between d and e; and $P < 0.001$ between d and f. Details regarding the activity determinations are given in Materials and Methods.

[c]Values were calculated relative to the control (without additives) using mean values.

TABLE 3

Substrate Specificity of Lipase Activity

Substrate[a]	Activity[b] (μmol/min/mg protein)	Relative activities (%)[c]
Triolein (18:1*cis*-9)	37.5 ± 1.0[e]	100
Trilinolein (18:2*cis*-9,12)	41.7 ± 0.6[f]	111
Trilinolenin (18:3*cis*-9,12,15)	45.8 ± 1.5[g]	122
Trielaidin (18:1*trans*-9)	6.8 ± 0.8[g]	18
Tristearin (18:0)[d]	0	0

[a]Triacylglycerols were emulsified in 5 mL deionized water with CHAPS (for abbreviation, see Table 2) (1.6 mM). Substrate concentration in the 10 mL reaction mixture was 20 mM for triolein, trilinolein, trilinolenin and trielaidin; and 5 to 10 mM for tristearin. CHAPS concentration in the reaction mixture was 0.8 mM. The reaction mixture (pH 8.0) was incubated at 30°C for 60 min, and the free fatty acids were titrated with 0.05 N NaOH as described in Materials and Methods.

[b]Each value is the mean ± SD of three determinations. Significance was assessed by Student's *t*-test; $P < 0.01$ between e and f, and $P < 0.001$ for e and g and f and g.

[c]Values were calculated relative to triolein using only the mean values.

[d]No titrable fatty acid was detected when either taurocholic acid (4 mM) or CHAPS (0.8 mM) were used as surfactants.

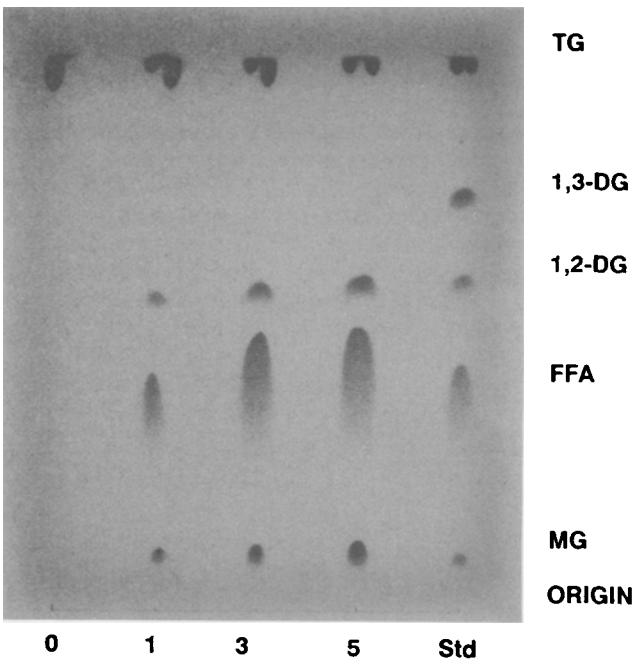

FIG. 3. Thin-layer chromatography of products of triolein hydrolysis by extracellular lipase from *Pythium ultimum*. The reaction conditions are given in the Materials and Methods section. TG, triacylglycerol; 1,3-DG, 1,3-diacylglycerol; 1,2-DG, 1,2-diacylglycerol; FFA, free fatty acid; MG, monoacylglycerol.

The rate of triacylglycerol hydrolysis increased with increasing number of double bonds per molecule. This result is unlike those reported previously with lipases from *C. deformans* and *H. lanuginosa* No. 3 for which the presence of two and especially three double bonds in the C_{18} fatty acid chains reduced the rate of hydrolysis (17,20). In contrast, an extracellular lipase from the fungus *G. candidum* can hydrolyze triacylglycerol containing 18:1 *cis*-9 and 18:2 *cis* 9,12 unsaturated fatty acids from natural and synthetic substrates regardless of their position within the substrate molecule (22). We cannot say unequivocally whether the differences in *Pythium* lipase activity with different substrates were due to differences in emulsion properties of substrates, or the substrate structure *per se*, but lipases isolated from various sources have been shown to exhibit different substrate preferences. For example, lipases from *N. crassa* readily hydrolyzed triacylglycerols with C_{16} and C_{18} fatty acids, hydrolyzed equally saturated and unsaturated substrates (18), but hydrolyzed triacylglycerols with short chain fatty acids (C_4-C_{10}) at a very slow rate. A lipase from *S. lipolytica* preferred substrates with short chain fatty acids and required oleic acid as an activator (7). Furthermore, multiple forms of lipases from the same fungus have been shown to exhibit different substrate preferences on the basis of either acyl chain length (12) or degree of unsaturation (16).

Positional specificity. Figure 3 shows the course of triolein hydrolysis by the lipase from *P. ultimum*. 1,2-Diolein was produced along with monoolein and oleic acid. 1,3-Diolein was not detected by TLC. It is clear from the TLC analysis of the reaction mixture that the lipase did not hydrolyze the ester bond at position 2 of triolein, indicating that the enzyme has 1,3-positional specificity which has been typical of the fungal lipases studied previously.

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Purification and Characterization of a Fatty Acid Binding Protein from Human Prostatic Tissue[1]

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Epidemiological studies suggest the existence of a strong relationship between the incidence of prostatic cancer and the intake of dietary lipids in humans. However, very little information is available on intracellular fatty acid metabolism in human prostatic tissue. The objective of this study was to identify and subsequently characterize a fatty acid binding protein of human prostatic tissue. A fatty acid binding protein (FABP) was purified and characterized from human prostatic tissue. The purified FABP had an apparent molecular mass of 15.0 ± 1.0 kDa as averaged from three different methods, sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel filtration and amino acid analysis. The pI value of the protein was determined to be 6.8. Scatchard analysis of fatty acid binding to the purified FABP from malignant prostatic tissue showed a Kd value of 0.53 ± 0.02 μM for arachidonic acid (n = 5). The Kd values of FABP purified from benign prostatic tissue were 0.57 ± 0.02 μM for oleic acid and 0.51± 0.04 μM for arachidonic acid (n = 5). Fatty acid analysis revealed that the level of endogenously bound arachidonic acid was about 2.5-fold higher in FABP from malignant than from benign tissue. In addition, both malignant and benign tissues contained the same concentration of FABP. The concentrations of FABP in malignant and benign tissues were 19.2 ± 1.8 and 21.4 ± 2.1 μg per mg of total cytosolic protein, respectively. Characterization based on amino acid composition, isoelectric point and fluorescence with dansyl undecanoic acid suggests that the FABP may not be of the heart type, but is rather more closely related to the liver type. As malignant prostatic tissue produces more PGE_2 compared to benign tissue, our data suggest that FABP may help enhancing the synthesis of the prostaglandin in malignant tissue by facilitating arachidonic acid transport.

Lipids 28, 383-388 (1993).

Fatty acid binding proteins (FABP) belong to a family of small molecular weight cytosolic proteins (14–15 kDa) which are found in abundance in a number of tissues of mammals, birds, fish and insects (2,3). Intracellular fatty acids are critical molecules for energy delivery and for the synthesis of membrane lipids and lipid mediators. Thus FABP may have regulatory functions in energy production as well as in the synthesis of membrane lipids and lipid mediators (prostaglandins, leukotrienes and thromboxanes). The significance of FABP in intracellular fatty acid metabolism has been demonstrated in several studies (2,3). FABP are also associated with cell growth and regulation by virtue of binding various growth inhibitory and stimulatory compounds (4). Liver-type FABP binds various eicosanoids such as prostaglandin E_1 (PGE_1) (5), the lipoxygenase metabolites of arachidonic acid (6) and growth inhibitory cyclopentenone prostaglandins such as $^{\Delta 12}$-PGJ_2 (9-deoxy $^{\Delta 9,12}$-13,14-dihydro-prostaglandin D_2) and prostaglandin A_2 (PGA_2) (7). FABP binds these cyclopentenone prostaglandins avidly and apparently transports them to the nuclear membrane (7,8). These prostaglandins induce *gadd* 153 mRNA, a member of a novel class of genes associated with growth arrest and DNA damage (9). In the phospholipids of malignant human prostatic tissue, only the arachidonic acid (20:4n-6) level was significantly lower compared to the level in benign tissue; no difference was observed in the levels of linoleic acid (18:2n-6) or dihomogammalinolenic acid (20:3n-6) (10). The reduced arachidonic acid level in phospholipids of malignant tissue may be due to increased synthesis of prostaglandin E_2 (PGE_2), which is reported to be a tumor promoter in prostatic tissue (11–13). FABP have been implicated in prostaglandin metabolism as well as in cell growth and regulation (4,14); however, no information is available on FABP in human prostatic tissue. Here we report the purification and characterization of a FABP from human prostatic tissue.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleic acid (53 mCi/mmol) and [5,6,8,9,11,12,14,15-^{3}H(n)]arachidonic acid (83.6 mCi/mmol) were obtained from New England Nuclear (Hertfordshire, United Kingdom). Sepharoses CL-4B, rabbit anti-human albumin IgG and unlabelled fatty acids were obtained from Sigma Chemicals (Poole, United Kingdom). The molecular weight protein standard kit, and pI marker proteins (pI 3.5 to 9.3) were obtained from Pharmacia-LKB Biotechnology (Uppsala, Sweden). 11-(Dansylamino)undecanoic acid (dansyl undecanoic acid) was obtained from Molecular Probes (Junction City, OR), and Lipidex-1000 was obtained from United Technologies Packard (Downers Grove, IL). All other reagents used were of analytical grade quality. Prostatic tissue was obtained from Aberdeen Royal Infirmary, Aberdeen, Scotland.

Preparation of FABP. Prostatic tissue was obtained from patients undergoing surgical removal of the prostate gland with a provisional diagnosis of benign or malignant prostatic disease. Prostatic tissue was collected fresh at operation and was immediately frozen in liquid nitrogen

[1]A preliminary account of this work was presented at the Biochemical Society Meeting, London, December 16-18, 1991, and published as an abstract (Ref. 1).

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Abbreviations: Dansyl undecanoic acid, 11-(dansylamino)undecanoic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EFA, essential fatty acid; FABP, fatty acid binding protein(s); FPLC, fast protein liquid chromatography; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoic acd; 5-HETE, 5-hydroxyeicosatetraenoic acid, PITC, phenylisothiocyanate; PMSF, phenylmethylsulfonyl fluoride; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; PGD_2; prostaglandin D_2; PGA_1, prostaglandin A_1; PGA_2, prostaglandin A_2; $^{\Delta 12}$-PGJ_2, 9-deoxy$^{\Delta 9,12}$-13,14-dihydro-prostaglandin D_2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

and then stored at −70°C until required. Samples were classified as benign or malignant by histological examination. FABP was purified separately from malignant and benign prostatic tissues following the procedures as described previously with slight modifications (5). Typically, 20 g prostatic tissue (pooled tissues either from benign or malignant samples) was washed twice with ice-cold saline to remove blood. The tissue was then blotted and weighed. After chopping, the tissue was homogenized in 70% (wt/vol) 20 mM Tris-Hcl buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.25 mM sucrose. After centrifugation at 5000 × *g* for 15 min, the pellet was discarded and the supernatant was then centrifuged at 110,000 × *g* for 1 h 20 min at 4°C. The supernatant obtained after the second centrifugation was subjected to 70% ammonium sulfate saturation, then centrifuged at 10,000 × *g* for 20 min. The supernatant was dialyzed against 10 mM Tris-HCl buffer containing 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.002% sodium azide at 4°C for 24 h and concentrated by ultrafiltration using a filter with a molecular weight cut-off of 3,500 daltons (Amicon Diaflow, Glucocestershire, United Kingdom). The dialyzed and concentrated fraction was termed fraction "A". FABP was then finally purified to homogeneity from fraction "A" by fast protein liquid chromatography (FPLC) (Pharmacia-LKB Biotechnology, Uppsala, Sweden) using Sephacryl S-300 and Superose 12 columns, respectively. The presence of FABP in protein samples at different stages of purification was monitored using the radiochemical binding assay as described elsewhere. Protein was detected in column fractions by measuring absorption at 280 nm.

The protein concentrations in the tissue homogenate or in the pooled column fractions were determined according to the Bradford method using bovine serum albumin as standard (15). The FABP concentrations calculated from quantitative amino acid analysis were used only for determination of B_{max} and K_d values of fatty acid binding. In some cases, it was necessary to delipidize the FABP preparation. Delipidation of protein was done by passing through a Lipidex-1000 column (0.5 × 8 cm) at 37°C, as described previously (16).

Sephacryl S-300 chromatography. All the chromatographic procedures described below were carried out at 4°C. Approximately 30 mg of proteins from de-albuminized fraction "A" were applied to an FPLC Sephacryl S-300 column (2.6 × 60 cm) which had previously been equilibrated with 10 mM Tris-HCl buffer, pH 8.2 containing 0.1 mM PMSF, 1 mM DTT, 1 mM EDTA and 0.002% sodium azide. The fractions containing FABP were eluted with the same buffer at a flow rate of 1 mL/min. The FABP fractions were then pooled (300–350 mL) and concentrated for further purification.

Superose 12 column chromatography. The FABP fractions eluted from the Sephacryl S-300 column still contained some high molecular weight proteins. Further purification was achieved by applying 5 mg of the eluted FABP to an FPLC Superose 12 column (26 × 1.6 cm) which had previously been equilibrated with the same buffer used in the Sephacryl S-300 column. The fractions containing fatty acid binding activity were eluted at 40–65 mL. They were pooled and concentrated for further studies.

Polyacrylamide gel electrophoresis and molecular mass determination. The purity of the FABP preparation at each step was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on SDS Phast gel homogeneous 20 (Phast System, Pharmacia-LKB Biotechnology). The relative molecular mass of FABP was determined with the aid of marker proteins. The purified protein was kept at −70°C for further study.

Isoelectrofocusing of FABP. Isoelectric focusing of the delipidized FABP was performed in the Phast system (Pharmacia-LKB Biotechnology) on a Phastgel IEF 3-9, exactly as described in the instrument manual.

Amino acid analysis. Amino acid analysis of the purified FABP was carried out using a Waters Pico-Tag Amino Acid Analyser (Millipore, Edinburgh, United Kingdom). Protein (100 μL) was hydrolyzed with 6.0 N HCl at 110°C for 18 h. The phenylisothiocyanate (PITC)-amino acids generated were identified using a C_{18} reverse-phase narrow bore cartridge. Norleucine was used as an internal standard (200 nmol/mL). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively.

The amino acid composition of proteins constitutes a set of integral residue numbers, whereas amino acid analysis gives nonintegral numbers. However, a minimum can be obtained in the function

$$y = \left[\sum_{i=1}^{n} \frac{|x_i - \text{nearest integer}|}{\text{nearest integer}} \cdot 100 \; \frac{1}{n} \right] \qquad [1]$$

which allows us to find the appropriate integers for each amino acid and to calculate the corresponding relative molecular mass (17). In the above equation, y (in %) represents the average deviation from the nearest integer of n different amino acids present in the protein, x_i is the non-integral residue number of amino acid i, calculated in relation to an arbitrary 'key' amino acid. To cover the prospective range of the relative molecular mass, a set of integer values is chosen for the key amino acid.

Fatty acid binding assay. The fatty acid binding assay was performed as described previously using the Lipidex method (5). Typically, 10–20 μg of protein in 50 mM Tris-HCl buffer, pH 7.4, was incubated with 1 μM [^{14}C]oleic acid, or [^{3}H]arachidonic acid, for 20 min at 37°C in a total volume of 200 μL. After incubation, the assay mixture was cooled and 100 μL of Lipidex-1000 suspension was added to remove the unbound radioligand. After further incubation for 10 min at 4°C, it was centrifuged. Radioactivity was determined in 100 μL of supernatant by liquid scintillation counting. Non-specific binding was determined in the presence of a 1000-fold excess of unlabelled fatty acids. For accurate determination of binding kinetics, Scatchard analysis was performed by incubating 15 μg of delipidated FABP in the presence of 100 μM Triton X-100 (18) in the above assay mixture with various concentrations of oleic acid or arachidonic acid (0.1–1 μM).

Cytosolic protein samples or column fractions were routinely tested for FABP contents at 1 μM [^{14}C]oleic acid concentration.

Fatty acid analysis. Endogenous fatty acids from FABP were extracted according to the method of Folch *et al.* (19). The fatty acid composition was then analyzed in a

Hewlett-Packard gas chromatograph (Hewlett-Packard, Geneva, Switzerland), after methylation of fatty acids by methanolic HCl as described previously (20).

Fluorescence assay. For the determination of FABP concentrations in benign and malignant prostatic tissue by the fluorescence method, tissues (average weight 2.23 g, n = 5) were separately processed and fraction "A" was prepared as described above. As albumin also binds dansyl undecanoic acid, albumin was removed by passing the samples through an affinity column of rabbit anti-human albumin IgG bound to Sepharose CL-4B after activation with tresyl chloride. Concentrations of FABP in these samples were measured by a fluorescence assay described previously (21). Dansyl undecanoic acid (0.1 mM) solution was prepared in 20 mM Tris-HCl buffer, pH 7.8. Fluorescence measurements were carried out in a Perkin-Elmer Luminescence spectrophotometer LS-5B (Perkin-Elmer, Buckinghamshire, United Kingdom) at 22°C. Fluorescence intensity was measured at 500 nm after excitation at 350 nm, with a slit width of 10 nm for both excitation and emission. Small aliquots (5 μL) of dansyl undecanoic acid stock solution (0.1 mM) were added by a micro syringe to the cuvettes containing either buffer or protein sample (3 mL). Maximum fluorescence enhancement for aliquots of de-albuminized fraction "A" was then determined. FABP concentration was estimated in these samples by using a standard curve prepared with purified FABP from prostate tissue, as described earlier (21,22).

Statistical analysis. Differences between the benign and malignant prostatic tissues were assessed by means of Student's unpaired *t*-test. Differences were considered significant when the *P* value was less than 0.05. Results are expressed as means ± SD.

RESULTS

Identification and purification of FABP from human prostatic tissue. The purification protocol of prostatic FABP is summarized in Table 1. FABP was purified to homogeneity both from benign and malignant human prostatic tissue as described in the Methods section. Figure 1 shows the elution profile of de-albuminized fraction "A" from Sephacryl S-300 column. FABP fractions emerged around 300–350 mL. Figure 2 shows an SDS-PAGE (15%) of the 70% ammonium sulfate fraction (supernatant) and FABP fractions obtained from the Superose 12 column. SDS-PAGE of the purified FABP protein showed a single band with a mobility that corresponded to a M_r 14,400. FABP was purified from both malignant or benign prostatic tissues, as described in the Methods section, yielding 0.51 ± 0.02 mg FABP/g wet tissue (n = 5).

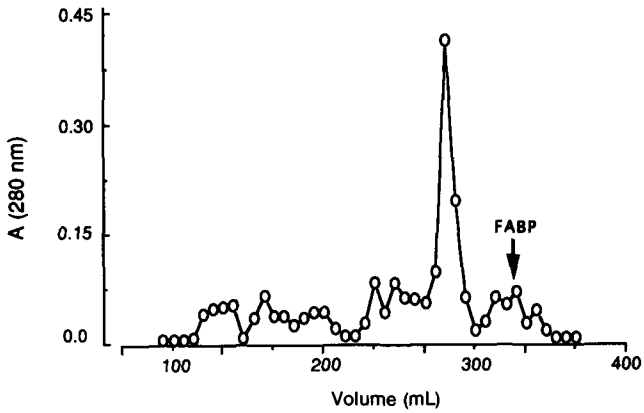

FIG. 1. Chromatography of prostate supernatant on a fast protein liquid chromatography (FPLC) Sephacryl S-300 column. De-albuminized fraction "A" (30 mg) was loaded onto an FPLC Sephacryl S-300 column equilibrated with 10 mM Tris-HCl, pH 8.2, containing 0.002% sodium azide and 1 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid. The proteins were eluted with the same buffer. The trace is representative of approximately 8 separations carried out. The fractions containing fatty acid binding activity are marked by fatty acid binding protein (FABP). FABP fractions were then pooled and purified further using a Superose 12 column.

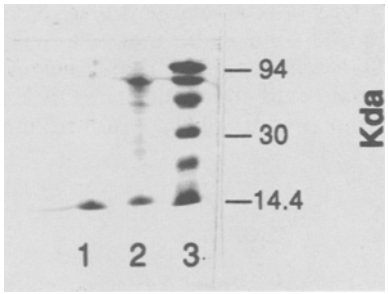

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins. Fractions were separated in an SDS-PAGE gel (20%) and stained for protein with coomassie blue. Lane 1, purified fatty acid binding protein eluted from Superose 12 column; Lane 2, 70% ammonium sulfate fraction; Lane 3, the molecular mass markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin, (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), lactalbumin (14.4 kDa).

Amino acid composition and molecular mass determination. The amino acid composition of FABP by individual determinations is given in Table 2. In this protein, 2 Cys,

TABLE 1

Summary of FABP Purification from Human Prostatic Tissues[a]

Purification step	Protein (mg)	Specific binding activity (nmol/mg)	Purification (fold)	Yield (%)
110,000 × *g* supernatant	538.8	0.3	1.0	100.0
70% $(NH_4)_2$ SO_4 fraction	145.7	0.8	2.5	69.2
Sephacryl S-300 column	21.6	5.8	16.8	67.9
Superose 12 column	10.1	12.9	37.5	70.3

[a]Protein was determined by the Bradford method (15). FABP, fatty acid binding protein(s).

TABLE 2

Amino Acid Composition of FABP from Malignant Prostate Tissue[a]

Amino acid residues	Number of residues
Asx	15
Glx	9
Ser	9
Gly	18
His	10
Arg	7
Thr	6
Ala	10
Pro	4
Tyr	5
Val	10
Met	1
Cys	2
Ile	7
Phe	6
Trp	n.d.
Lys	7

[a]n.d., Not determined. See Table 1 for abbreviation.

1 Met, 6 Phe, 7 Arg and large numbers of Lys, Leu, Val and Pro residues were present (Table 2). Trp was not determined. An estimation of the M_r value of FABP from amino acid analysis by reduction of the residues using Leu, Thr, Gly and Asx as key amino acids gave average value of 14,895. The molecular mass was calculated to be 15.0 ± 1.0 kDa when averaged from three different determinations (amino acid composition, SDS-PAGE and gel filtration). The procedure of the Bradford method gave a value of protein mass which was 1.4- to 1.6-fold lower than that determined by quantitative amino acid analysis. Isoelectric focusing of the purified FABP also revealed a single band with a pI value of 6.85 (results not shown).

Fatty acid binding activity. Prufied FABP from malignant and benign tissues showed similar binding kinetics toward arachidonic and oleic acids. Scatchard analysis showed a Kd value, for FABP from benign prostate tissue, of 0.57 ± 0.01 μM for oleic acid and 0.51 ± 0.04 μM for arachidonic acid (n = 5), whereas the Kd values for FABP from malignant tissue were 0.53 ± 0.02 μM for oleic acid and 0.44 ± 0.01 μM for arachidonic acid (n = 5) (Table 3). The maximal binding capacities (B_{max}) for oleic acid and arachidonic acid were 1.4 and 1.7 mol per mol of FABP, respectively.

Fatty acid analysis. The composition of endogenously bound fatty acids from purified FABP from malignant and benign tissues is shown in Table 4. No significant difference was observed in the levels of individual fatty acids except the level of arachidonic acid which was 2.5-fold greater in FABP of malignant tissue compared to that of benign tissue. Malignant prostatic tissue FABP contained 4.6 nmoles of arachidonic acid per mg of protein as compared to 1.8 nmoles arachidonic acid per mg of protein in FABP of benign tissue ($P < 0.05$, *t*-test). However, the total endogenous fatty acid bound to FABP in these two tissues did not differ significantly, 78.1 and 76.0 nmoles per mg FABP ($P > 0.623$, *t*-test, n = 5) in malignant and benign tissues, respectively.

Quantitation of FABP in benign and malignant human prostatic tissue. Dansyl undecanoic acid was used as a fluorescence probe to determine FABP levels in the fraction "A". Interaction of dansyl undecanoic acid with

TABLE 3

Scatchard Analysis of Fatty Acid Binding to Prostatic FABP[a]

	FABP (malignant tissue)		FABP (benigh tissue)	
Fatty acids	Kd (μM)	B_{max} (mol/mol)	Kd (μM)	B_{max} (mol/mol)
Oleic acid	0.53 ± 0.02	1.42 ± 0.34	0.57 ± 0.01	1.52 ± 0.23
Arachidonic acid	0.44 ± 0.01	1.65 ± 0.39	0.51 ± 0.04	1.72 ± 0.27

[a]The binding values were determined from Scatchard individual isotherms. The amount of prostatic FABP used was approximately 15 μg (n = 5). See Table 1 for abbreviation.

TABLE 4

Endogenous Fatty Acids Associated with FABP Purified from Benign and Malignant Human Prostatic Tissues[a]

	(nmol per mg of protein)	
Fatty acid	FABP (malignant tissue)	FABP (benign tissue)
16:0	18.4 ± 2.4	17.4 ± 1.7
18:0	7.1 ± 1.4	6.5 ± 1.2
18:1n-9	20.4 ± 3.2	24.8 ± 2.9
18:2n-6	26.8 ± 4.0	24.4 ± 3.4
18:3n-6	0.6 ± 0.0	0.7 ± 0.0
20:3n-6	0.2 ± 0.0	0.1 ± 0.0
20:4n-6	4.6 ± 0.9	1.8 ± 0.0[b]

[a]Each value is the mean ± SD (n = 5). See Table 1 for abbreviation.
[b]Significantly different from the malignant, $P < 0.05$, Student's *t*-test.

prostate FABP caused a large fluorescence enhancement and a considerable shift both in the excitation maxima (from 330 to 350 nm) and emission maxima (550 to 500 nm) wavelength (data not shown). FABP concentrations in benign and malignant prostatic tissues were estimated to be 19 ± 1.8 and 21 ± 2.1 μg per mg of cytosolic protein ($P > 0.534$, n = 5), respectively, as measured in "fraction A" of these samples using the fluorescence assay described in the Methods section. The validity of this method was confirmed by measuring the total FABP content in fraction "A" after adding known amounts of pure FABP. The accuracy of quantitation of FABP by this method was found to be within 95-97%.

DISCUSSION

Our data demonstrate for the first time the presence of an FABP in human prostatic tissue. The FABP was purified and characterized from both benign and malignant prostatic tissue. The relative molecular mass of the purified FABP was calculated to be 15.0 ± 1.0 kDa as derived from three independent methods (SDS-PAGE, gel filtration and from amino acid analysis). The average molecular mass of prostate FABP is similar to the size of FABP types from other species (2,3). No significant difference was observed in FABP concentrations between malignant and benign prostatic tissue. The *N*-terminal of this protein was found to be blocked; therefore sequencing was precluded. The relative excitation maxima at 274 nm and emission maxima at 309 nm are due to tyrosine residues, while the small peak at 258 nm reflects the presence of phenylalanine residues (data not shown). The fluorescence analysis revealed that FABP does not have a tryptophan moiety as is also the case for liver FABP (2). Amino acid analysis of the FABP revealed the presence of one Met, two Cys, six residues of Phe and a large number of His and Arg residues.

So far, three different FABP types have been identified and characterized in man: liver, heart and intestinal FABP (23-25). The FABP types are reported to differ markedly in their isoelectric point, amino acid composition, fluorescence with dansyl undecanoic acid and immunological cross-reactivities (3). Our data on amino acid composition, pI value and the fluorescence with dansyl undecanoic acid suggest that prostatic FABP may not be of the heart type. The pI value of prostate FABP is 6.85 which is similar to liver-type FABP (neutral pI value), whereas heart-type FABP are acidic proteins (pI about 5.0) (2). The amino acid composition of prostatic FABP also clearly differs from the heart-type FABP, the major differences being the presence of two Cys residues in prostate FABP, which are absent in heart-type FABP (2). Furthermore, the absence of Trp in prostate FABP suggests that it may be more related to liver-type, which is devoid of Trp residue (3). Fluorescence data on the interaction of prostatic FABP with dansyl undecanoic acid showed a marked shift of both excitation and emission wavelength and a large fluorescence enhancement which are similar to those observed with liver-type FABP but not with heart-type FABP (21,22). However, further work on immunochemical reactivities of prostatic FABP against anti-sera of known human FABP types, as well as amino acid/cDNA sequence analysis of the protein or Northern blotting of mRNA, is required for definitive conclusions.

The binding kinetics showed no difference in the Kd values of FABP of malignant and benign prostatic tissues for oleic acid and arachidonic acid. The values of the same order of magnitudes as the Kd are described for other FABP types (2). A discrepancy is usually observed with the stoichiometry of ligand binding by FABP. In our experiments, prostate FABP appears to bind more than one mole of oleic or arachidonic acid (1.4 to 1.7 mol fatty acid per mol FABP). It has been generally accepted that FABP bind one mole of fatty acid per mol of protein (2). Liver-type FABP has a binding stoichiometry which appears to vary between 1 and 3 (23,26-28). The differences in B_{max} value may be due to variations in procedures employed for the radiochemical binding assay, preparations of FABP, as well as for the ligand preparation.

Prostate FABP endogenously bound 76 to 78 nmoles fatty acids per mg of protein which is equivalent to 1 mol fatty acid bound per mol FABP. *In vivo*, the protein would be fully loaded with ligands only if the B_{max} is around one mole fatty acid per mol of FABP. However, the B_{max} of the prostate FABP appears to be 1.4 to 1.7 mol per mol protein. The molar ratio of endogenously bound fatty acids is reported to vary between 0.4 and 1.3 mol per mol of FABP (2). The isolation procedures for FABP may also have effects on the quantity of endogenously bound fatty acids. This variation could also be due to the results of lipolysis before or during the homogenization of tissue. Although the prostatic tissue used for FABP preparation was immediately frozen after surgical removal, it is very unlikely that freezing will stop tissue lipolysis completely. Despite the similar affinity for arachidonate, the endogenous level of this fatty acid in FABP of malignant tissue was found to be 2.5-fold higher than that of benign tissue, possibly indicating an increased level of free arachidonic acid in the cytoplasm of malignant tissue compared to benign tissue. FABP isolated from various tissues differ both qualitatively and quantitatively in bound long-chain fatty acids. In the present case, differences may be the result of higher activity of phospholipase(s) in malignant prostatic tissue compared to benign tissue. Alterations of essential fatty acid (EFA) and prostaglandin metabolism in malignant human tissues have been reported (10,29,30). Whether profiles of endogenously bound fatty acids to FABP reflect the status of intracellular fatty acid and prostaglandin metabolism in malignant and benign tissues remains to be established. In any event, our data show that more arachidonic acid is endogenously bound to FABP of malignant tissue compared to benign tissue. The increased arachidonic acid bound to FABP of malignant prostate tissue and the reduced arachidonic acid levels in phospholipids of malignant tissue compared to benign tissue (10) may suggest a difference in the degree of phospholipase activity in these tissues. Increased prostaglandin synthesis in the kidney of genetically hypertensive rats is thought to be facilitated by increased levels of arachidonic acid bound to FABP (14). The synthesis of prostaglandins depends on the availability of substrate. However, it is tempting to speculate that FABP in malignant prostatic tissue may help to enhance prostaglandin synthesis by transporting more arachidonic acid to the cyclooxygenase in the endoplasmic reticulum. Whether FABP enhance prostaglandin synthesis by increasing arachidonic acid transport needs further investigation. Preliminary studies showed that malignant prostatic

tissue produced 12-fold more PGE_2 than the benign tissue when these tissues were incubated with radiolabelled arachidonic acid (31).

In addition to fatty acid binding, liver-type FABP also binds PGE_1, growth inhibitory cyclopentenone prostaglandins (Δ^{12}-PGJ_2, PGA_1 and PGA_2) and growth stimulatory lipoxygenase metabolites (15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetrenoic acid, 5-HETE, 5-hydroxyeicosatetraenoic acid), with similar affinities (Kd in nanomolar range) (5-7). However, the implication of these observations in cell growth and regulation is not yet elucidated. Since prostate FABP appears to be closely related to the liver-type FABP, future studies on its role in intracellular eicosanoid metabolism and transport in prostate tissue should give us a better understanding of the precise function of FABP in cellular growth and regulation.

In conclusion, we have identified and purified FABP from benign and malignant human prostatic tissue. The prostatic FABP seem to be more related to liver-type FABP with respect to amino acid composition, electrophoretic behavior and fluorescence data, but its precise role remains to be established.

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Trans-Monoenoic and Polyunsaturated Fatty Acids in Phospholipids of a *Vibrio* Species of Bacterium in Relation to Growth Conditions

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A *Vibrio* species of bacterium known to contain the polyunsaturated fatty acid 20:5n-3 was grown in both freshwater and seawater media at 5 and 20°C and examined for adaptive changes in lipid composition. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), together with a smaller proportion of nonesterified fatty acids (NEFA), comprised almost all the lipid under all growth conditions examined. Temperature had a more pronounced effect than the salinity of the medium on lipid composition. The proportion of PE in total lipid was always higher at 5 than at 20°C. Conversely, the proportion of NEFA was lower at 5 than 20°C whereas that of PG was not altered. The levels of saturated fatty acids in total lipid, PE and PG were all decreased by growth at 5°C. No differences were observed with respect to growth temperature in the levels of *cis* 16:1n-7, the principal monoenoic fatty acid in both PE and PG. *Trans* 16:1n-7 was found to comprise 12.8–15.2% of fatty acids in PE and PG of bacteria grown at 5°C but only 4.4–8.5% of phospholipid fatty acids in bacteria cultured at 20°C. Regardless of medium composition, a reduction in growth temperature from 20 to 5°C also caused the proportions of 20:5n-3 to increase from around 0.8 to 4.4% in PE and from around 4 to 20% in PG. The simultaneous occurrence of *trans* 16:1n-7 and 20:5n-3 is unique to this *Vibrio* species of bacterium. The increased proportions of both these fatty acids with decreasing temperature suggest that they have a role in retailoring biomembrane phospholipids during temperature acclimation of the bacterium.

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In general, the lipids of bacteria do not contain polyunsaturated fatty acids (1). An exception to this rule is the occurrence of polyunsaturated fatty acids in a few species of marine bacteria (2–5) isolated from diverse sources including seawater, deep sea sediments and the intestines of fish. These bacteria are invariably motile and Gram-negative, with *Vibrio* species being the most common (4,5).

Studies to date on the lipids of these bacteria which contain polyunsaturated fatty acids have examined only their total lipid and have not reported the distribution of individual fatty acids in the component lipid classes. Furthermore, although the biosynthesis of monounsaturated fatty acids has been well studied in bacteria (1), nothing is known of the mechanisms of bacterial synthesis of polyunsaturated fatty acids.

An increase in the level of unsaturated fatty acids in phospholipids of eukaryotic poikilotherms, both invertebrate and vertebrate, has been shown frequently to occur as an adaptation to a decrease in environmental temperature (6–8). In contrast, the effect of changes in environmental temperature on the detailed lipid composition of bacteria has been less well studied. The few studies that have been carried out show that there is no uniformity between different bacterial species in the changes that occur in lipid composition in response to changes in growth temperature. For example, in marine *Vibrios*, increases in the proportion of monounsaturated fatty acids, or increased synthesis of shorter chain fatty acids or no changes at all have all been observed as responses to reduced growth temperature (9). Most recently, the interconversion of *cis* and *trans* isomers of 16:1n-7 has been implicated in the thermal adaptation of a psychrophilic species of *Vibrio* (10,11).

In addition to growth temperature, the salt concentration of the medium may also influence the lipid composition of bacteria. This aspect has been studied in several species of bacteria including species of *Paracoccus* (12), *Staphylococcus* (13,14), *Vibrio* (15) and *Pseudomonas* (16). With Gram-negative bacteria in general, the proportions of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) increase and decrease, respectively, with increasing salt concentration in the growth medium (12,15, 16). However, the effect is somewhat different with the Gram-positive *Staphylococcus* species in that the PG decreases with increasing salinity while cardiolipin increases (13,14). Changes in fatty acid composition of total lipid are also observed in relation to the salinity of the growth medium. Thus, the level of monounsaturated fatty acids in total lipid has been shown to decrease significantly when marine species of *Vibrio* are grown in medium of reduced salinity (17) whereas with the moderate halophile *Vibrio costicola* the relationship between salt concentration and the proportion of monounsaturated fatty acids is parabolic with a maximum at 1.0 M (18).

To establish whether adaptive changes occur in the lipid composition of bacteria which are capable of producing polyunsaturated fatty acids, the lipids synthesized by a *Vibrio* species known to contain 20:5n-3 were examined in relation to growth conditions.

MATERIALS AND METHODS

Chemicals. Lipid standards were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom) and Supelchem U.K. Ltd. (Saffron Walden, United Kingdom). Solvents were of glass distilled or high-performance liquid chromatography (HPLC) grade and were supplied by Rathburn Chemicals (Walkerburn, United Kingdom).

Organism and growth conditions. The *Vibrio* species used in the study was originally isolated as described elsewhere (19) from the gut of Arctic char (*Salvelinus alpinus* L.) maintained in freshwater. To remove lipid from growth media, Oxoid general purpose nutrient broth powder (Unipath Ltd., Basingstoke, United Kingdom) was extracted with chloroform/methanol (2:1, vol/vol), filtered and air dried before being used for the preparation of the

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; NEFA, nonesterified fatty acids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; R_f, retardation factor; Tris, tris(hydroxymethyl)aminomethane.

media. The defatted nutrient broth was made up at a concentration of 13 g/L either in distilled water or artificial seawater (approximately 0.4M NaCl) buffered with 1.6 mM Tris HCl to produce freshwater and seawater medium, respectively. The pH of both media was 7.2. Cultures were grown in the dark under sterile conditions in glass conical flasks in temperature-controlled shaking incubators at either 5 or 20°C. Growth rates were assessed by measurement of absorbance at 600 nm against non-inoculated medium as the blank. Bacterial cells were harvested from cultures grown at 20 and 5°C after 24 h and 5 d, respectively, by centrifugation at 7,000 × *g* for 10 min. The resulting pellet was resuspended in the washing buffer of Hanna *et al.* (18) and the centrifugation repeated. Pellets of cells were lyophilized and weighed prior to lipid extraction.

Lipid analysis. Lipid was extracted by blending pelleted cells with chloroform/propan-2-ol (2:1, vol/vol) in a glass/Teflon homogenizer. The organic phase was then filtered and washed with one-quarter its volume of 0.88% (wt/vol) KCl. After removal of the solvent by evaporation under nitrogen, the lipid extract was desiccated overnight *in vacuo.* After weighing, the lipid extract was redissolved in a known volume of chloroform/methanol (2:1, vol/vol) and stored at −70°C until analyzed further.

Lipid class analyses were performed by high-performance thin-layer chromatography (HPTLC) using various solvent systems. Separated lipid classes were identified by reference to authentic standards and by the staining of chromatograms with reagents specific for functional groups (20). Two-dimensional HPTLC was performed using methyl acetate/propan-2-ol/chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol) and chloroform/methanol/7M aqueous ammonia (65:35:5, by vol) for development in the first and second dimensions, respectively. For quantitation of lipid classes, plates were developed to two-thirds of their length in chloroform/methanol/water (65:25:4, by vol) followed, after drying of the plate, by development to full distance with hexane/diethyl ether/acetic acid (90:10:1, by vol). Developed chromatograms were sprayed with 3% (wt/vol) copper acetate in 8% (vol/vol) phosphoric acid and the stained classes quantitated by scanning densitometry using a Shimadzu (Kyoto, Japan) CS9000 dual wavelength scanner attached to a Shimadzu DR-13 data recorder as described elsewhere (21).

For analysis of their constituent fatty acids, lipid classes were separated by HPTLC using the double development solvent system described above. Developed chromatograms were sprayed lightly with (0.01% wt/vol) 2′,7′-dichlorofluorescein in methanol and viewed under ultraviolet light. Bands of adsorbent containing the required classes were scraped from the plates and subjected to acid-catalyzed transesterification to produce the methyl ester derivatives of the constituent fatty acids (20). Fatty acid methyl esters were purified by HPTLC on silica gel plates using hexane/diethyl ether/glacial acetic acid (85:15:1, by vol) as developing solvent and recovered from the adsorbent by elution with hexane/diethyl ether (1:1, vol/vol). Aliquots of total lipid were also subjected directly to the acid-catalyzed transesterification procedure.

The analyses of fatty acid methyl esters of total lipid were performed using a Packard (Pangbourne, Berks., United Kingdom) 436 gas chromatograph equipped with a CP Wax 51 fused silica capillary column (50 m × 0.34 mm i.d.) (Chrompack UK Ltd., Milharbour, London, United Kingdom), on-column injection and H_2 as carrier gas. The oven temperature was programmed to increase from 50 to 225°C during the course of an analysis. For the analysis of fatty acid methyl esters of individual phospholipid classes, a fused silica capillary column coated with BPX 70 (25 m × 0.32 mm i.d.) was used, and the oven temperature was programmed to rise from 50 to 180°C. These samples were further analyzed using a chemically bonded CP Sil 5CB fused silica capillary column (50 m × 0.34 mm i.d.) with a single step thermal gradient from 50 to 260°C at 4°/min.

Separated methyl esters were identified by comparison with known standards and were quantitated using a Shimadzu CR-3A recording integrator attached to the gas chromatograph. The unsaturated nature of component methyl esters was confirmed by hydrogenation using PtO_2 followed by re-analysis. Identities were confirmed by gas chromatography/mass spectrometry (GC/MS) of selected samples by Dr. W.W. Christie, Hannah Research Institute, Ayr, United Kingdom, using conditions described elsewhere (22).

To confirm the identity of *trans* 16:1n-7, the fatty acid methyl esters of representative samples were separated on HPTLC plates impregnated with silver nitrate using hexane/diethyl ether/glacial acetic (94:4:2, by vol) as the developing solvent system (23). Components corresponding to saturated, *cis*-monounsaturated and *trans*-monounsaturated fatty acid methyl esters [identified by comparison of retardation factor (R_f) values with those of authentic standards run on the same chromatogram] were recovered from the adsorbent and analyzed by GC using the BPX70 phase as described above. The methyl ester of *trans* 16:1 was isolated as a component having an R_f value between that of saturated and *cis*-monounsaturated fatty acids.

Further confirmation of the identity of *trans* 16:1n-7 was obtained by stereospecific epoxidation with peracetic acid of fatty acid methyl esters separated by argentation chromatography (24). The epoxy derivatives were analyzed by GC using the BPX70 capillary column described above. The oven temperature was programmed to increase from the injection temperature of 50 to 150°C over 2.4 min and then after 18 min to increase to 220°C at 3 min. The separated epoxy derivatives of the methyl esters of the *cis* and *trans* isomers of 16:1n-7 in samples were identified by comparison of retention times of derivatives synthesized using standards of these fatty acids.

Student's *t*-test was used to determine the significance of differences between pairs of particular means (25), and differences were considered significant when $P < 0.05$. For the sake of clarity, values which are significantly different are not denoted in tables but are referred to in the text. The effects of the two factors (temperature and medium composition) were assessed by means of a two-way analysis of variance at a confidence level of 95% ($P < 0.05$) (25).

RESULTS

Growth characteristics. The *Vibrio* was capable of growing in both freshwater and seawater media. Growth rates, as assessed by absorbance measurements, were much higher at 20 than 5°C (Fig. 1) in both types of media. At 20°C the growth rates of bacteria grown in freshwater and

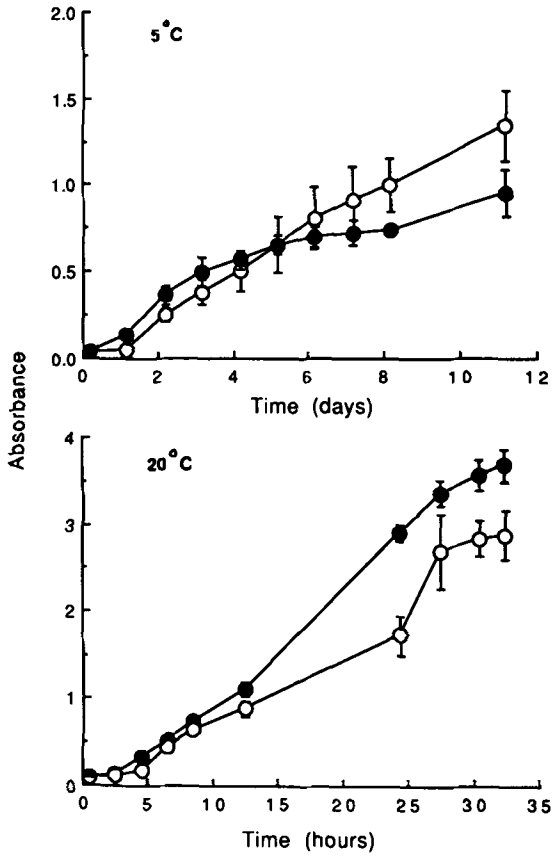

FIG. 1. Growth rates of *Vibrio* sp. cultured at 5 and 20°C in freshwater and seawater media. O, Seawater medium; ●, freshwater medium. Note different scales on x-axis.

seawater media were initially similar but diverged as the culture aged so that cultures grown in seawater medium finally contained fewer cells. The growth rate of bacteria at 5°C was higher in the freshwater medium than seawater medium over the initial 4 d of culture; but thereafter a higher biomass was observed in the seawater medium. Nevertheless, the influence of media composition on bacterial growth in Figure 1 was not marked. In contrast, further experiments showed that the *Vibrio* had poor growth rates in media containing 1M or 1.5M NaCl (data not shown).

Lipid content. Bacteria growth in seawater medium at 20°C contained only 4.5% of their dry weight as lipid whereas this component accounted for 7.4–7.9% of the dry weight of bacteria cultured under the other three conditions examined (Table 1). However, the only significant difference in lipid content was that observed between bacteria grown in seawater medium and bacteria grown in freshwater medium at 20°C. No statistically significant differences in lipid content existed between bacterial cultures in relation to growth temperature.

Lipid class composition. The lipid of the bacteria in all cultures was composed mainly of PE and PG (Table 1). At a given temperature (5 or 20°C) the lipid class composition was very similar for cells grown either in freshwater or seawater media. In bacteria grown at 5°C, more than 50% of the total lipid was PE, with PG and nonesterified fatty acids comprising around 31 and 12%, respectively, while at 20°C in both media the percentage of PE was substantially lower, and that of nonesterified fatty acids much higher, than at 5°C. The proportion of PG was only slightly higher in cells grown at 20°C than in those grown at 5°C in either medium. Overall, temperature affected lipid class composition more than the nature of the medium. LysoPE was present in trace amounts in lipid extracted from all cultures. When, in preliminary studies, chloroform/methanol (2:1, vol/vol) was used as extraction solvent, the levels of nonesterified fatty acids in the total lipid were notably higher, and those of PE and PG correspondingly lower (data not shown), than the values presented in Table 1. No components were observed in the total lipid extract which gave a positive stain for carbohydrate moieties.

Fatty acid composition of total lipid. The fatty acid compositions of total lipid from the *Vibrio* grown at 5 and 20°C in freshwater and seawater media are presented in Table 2. The major fatty acid component of total lipid was always the monounsaturated 16:1, but under the conditions employed complete resolution of the component *cis* and *trans* isomers of 16:1 was not attainable using the CP Wax 51 column. Overall, monounsaturated fatty acids accounted for just over half of the total fatty acids present

TABLE 1

Effect of Growth Temperature and Medium Composition on Lipid Content and Lipid Class Composition of *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
Lipid content (% dry weight)	7.90 ± 2.19	7.36 ± 0.50	4.53 ± 1.45	7.80 ± 0.60	—	—	0.0446
Lipid class composition (% total lipid)							
PE	55.6 ± 3.7	53.0 ± 1.6	38.9 ± 1.7	38.7 ± 4.2	0.0000	—	—
PG	31.1 ± 1.3	34.5 ± 3.1	36.3 ± 0.5	38.8 ± 1.6	0.0024	0.0278	—
NEFA	13.3 ± 3.0	12.3 ± 1.6	24.7 ± 2.0	22.4 ± 3.5	0.0001	—	—

[a]Values are means ± SD obtained with three cultures.

[b]Interaction between temperature and medium composition. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; NEFA, nonesterified fatty acids; —, not significant.

TABLE 2

Effect of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Total Lipid from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.5 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	—	—	—
12:0	4.0 ± 0.4	2.0 ± 1.7	4.3 ± 0.3	3.4 ± 0.3	—	0.0103	—
i-14:0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	—	—	—
14:0	2.9 ± 0.1	4.3 ± 0.2	2.4 ± 0.2	1.8 ± 0.2	0.0000	0.0324	0.0000
14:1n-5	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.0000	—	—
i-15:0	10.9 ± 0.4	12.6 ± 0.4	11.6 ± 0.5	15.4 ± 2.0	0.0200	0.0019	—
a-15:0	0.5 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.3	—	—	—
15:0	3.1 ± 0.2	2.2 ± 0.3	5.2 ± 0.2	5.8 ± 1.5	0.0001	—	—
15:1n-6	2.1 ± 0.5	1.0 ± 0.2	1.9 ± 0.1	3.0 ± 1.4	0.0420	—	0.0230
16:0	10.4 ± 0.3	13.3 ± 0.5	14.5 ± 1.8	13.3 ± 0.3	0.0042	—	0.0051
16:1	38.0 ± 1.0	39.8 ± 1.6	31.7 ± 0.7	28.9 ± 0.6	0.0000	—	0.0182
i-17:0	0.6 ± 0.2	0.6 ± 0.2	1.1 ± 0.1	1.4 ± 0.4	0.0033	—	—
17:0	0.7 ± 0.1	0.4 ± 0.1	2.7 ± 0.8	2.9 ± 0.2	0.0000	—	—
17:1n-8	6.7 ± 0.3	3.1 ± 0.4	10.6 ± 0.8	10.7 ± 1.9	0.0000	0.0052	0.0044
17:1n-6	1.4 ± 0.1	0.5 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	0.0030	0.0030	0.0003
18:0	1.1 ± 0.4	1.4 ± 0.4	1.2 ± 0.5	1.3 ± 0.2	—	—	—
18:1n-9	1.7 ± 0.1	1.7 ± 0.2	3.0 ± 0.4	2.7 ± 0.4	0.0002	—	—
18:1n-7	4.2 ± 0.1	3.8 ± 0.4	3.9 ± 0.3	4.4 ± 0.9	—	—	—
19:1n-8	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.1	—	—	—
20:5n-3	9.0 ± 0.7	9.0 ± 0.2	2.1 ± 0.6	1.9 ± 0.2	0.0000	—	—
22:0	0.2 ± 0.1	0.2 ± 0.1	ND	ND	—	—	—
Total sat.	35.3 ± 0.3	39.6 ± 0.8	44.3 ± 2.5	46.3 ± 1.6	0.0000	0.0073	—
Total mono.	55.3 ± 1.0	50.8 ± 0.6	52.8 ± 2.0	51.8 ± 2.0	—	0.0137	—
Total PUFA	9.0 ± 0.7	9.0 ± 0.2	2.1 ± 0.6	1.9 ± 0.2	0.0000	—	—
Unidentified	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.4	ND			

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

in the bacterial lipid regardless of growth conditions. An increase in the level of 16:1 with the lowering of growth temperature was largely balanced by reductions in the proportions of 17:1n-8 and 18:1n-9, and consequently the overall proportion of monounsaturated fatty acids in total lipid was not greatly affected by growth temperature. The proportions of saturated fatty acids in total lipid were significantly higher in bacteria grown in seawater and freshwater medium at 20°C than in those grown at 5°C. Palmitic acid (16:0) and *iso*-15:0 were the predominant saturated components present although smaller proportions of other saturated fatty acids, both with even and odd numbered chains, were also present. Under the conditions employed for the analysis of total lipid fatty acid methyl esters, any 13:0 and *iso*-13:0 present in the total lipid were obscured by the butylated hydroxytoluene added as antioxidant. The only polyunsaturated fatty acid present in the bacteria was 20:5n-3 which accounted for 9% of the fatty acids of total lipid from cultures grown at 5°C and around 2% of those from cultures grown at 20°C in either medium.

In contrast to temperature, the salt content of the medium had only a small effect on the fatty acid composition of total lipid at both temperatures examined. Small but significant differences in the levels of several fatty acids were notable between bacteria grown at 5°C in seawater and freshwater, with the overall result being higher and lower proportions of monounsaturated and saturated fatty acids, respectively, in the total lipid of bacteria grown in seawater medium. At 20°C, however, there were no overall changes in the levels of total saturated, monounsaturated or polyunsaturated fatty acids in the total lipids in relation to the salinity of the medium. Two-way analysis of variance with pooled means showed that, overall, temperatures had a greater effect on the fatty acid composition of total lipid than medium composition. The levels of 14:0, 15:1, 16:0, 16:1, 17:1n-8 and 17:1n-6 were also influenced significantly by the interaction of temperature and medium composition. That is to say, changing temperature and medium composition simultaneously brought about a significant change in the proportions of these components.

Fatty acid composition of lipid classes. The use of two columns for the analysis of fatty acid methyl esters of phospholipid classes allowed the resolution of more components than were obtained for total lipid samples. Thus 13:0 and *iso*-13:0, as well as the various isomers of 16:1, were discernable.

Although the fatty acid composition of PE (Table 3) showed general similarities with that of total lipid, it was notable that the proportions of polyunsaturated fatty acids in the phospholipid were only half those present in total lipid (Table 2) while those of total saturated fatty acids were higher under all growth conditions examined. The overall levels of monoenoic fatty acids in PE were only slightly lower than those observed in total lipid. *Iso*-15:0 and 16:0 were always the major saturated fatty acids in PE. Although the proportion of *iso*-15:0 in bacteria grown

TABLE 3

Effect of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Phosphatidylethanolamine from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	ND	—	—	—
12:0	3.8 ± 1.2	5.1 ± 0.6	3.7 ± 0.4	3.4 ± 0.3	—	—	—
i-13:0	4.5 ± 0.1	5.2 ± 0.6	3.8 ± 0.3	3.6 ± 0.4	0.0007	—	—
13:0	0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	—	—	—
i-14:0	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	0.0041	—	—
14:0	3.1 ± 0.3	4.7 ± 0.2	2.6 ± 0.2	1.7 ± 0.1	0.0000	0.0283	0.0000
14:1n-5	0.4 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.0008	—	—
i-15:0	14.2 ± 1.1	15.7 ± 0.4	13.5 ± 1.0	19.1 ± 1.8	—	0.0005	0.0149
a-15:0	0.2 ± 0.2	0.5 ± 0.3	0.2 ± 0.1	0.4 ± 0.4	—	—	—
15:0	3.5 ± 0.2	2.2 ± 0.4	5.5 ± 0.3	5.6 ± 1.3	0.0001	—	—
15:1n-6	2.5 ± 0.1	1.7 ± 0.7	1.8 ± 0.1	2.1 ± 0.6	—	0.0197	0.0033
16:0	10.5 ± 0.6	12.4 ± 0.4	14.6 ± 0.6	13.0 ± 0.9	0.0003	—	0.0010
trans 16:1n-7	12.8 ± 0.7	15.4 ± 0.7	4.4 ± 2.2	6.3 ± 0.8	0.0000	0.0420	—
16:1n-9	1.8 ± 0.1	1.9 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	0.0010	—	—
cis 16:1n-7	23.3 ± 0.6	21.1 ± 0.8	23.1 ± 5.5	18.0 ± 3.4	—	0.0187	—
i-17:0	0.3 ± 0.2	0.4 ± 0.2	0.9 ± 0.1	1.4 ± 0.4	0.0020	—	—
17:0	0.7 ± 0.1	0.4 ± 0.1	2.6 ± 0.4	2.6 ± 0.3	0.0000	—	—
17:1n-8	6.4 ± 0.3	2.7 ± 0.4	10.2 ± 0.3	9.4 ± 2.1	0.0000	0.0024	0.0128
17:1n-6	1.4 ± 0.3	0.5 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	0.0065	0.0096	0.0003
18:0	0.4 ± 0.1	0.4 ± 0.2	1.2 ± 0.4	1.0 ± 0.3	0.0015	—	—
18:1n-9	1.1 ± 0.1	1.0 ± 0.2	2.6 ± 0.5	2.1 ± 0.3	0.0000	—	—
18:1n-7	3.9 ± 0.5	3.4 ± 0.5	3.5 ± 0.3	4.3 ± 1.0	—	—	—
19:1n-8	0.5 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	—	—	—
20:5n-3	4.2 ± 0.4	4.4 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.0000	—	—
Total sat.	41.7 ± 1.7	47.3 ± 0.9	49.4 ± 3.1	52.7 ± 0.2	0.0001	0.0014	—
Total mono.	54.1 ± 1.5	48.3 ± 1.0	49.7 ± 2.7	46.5 ± 0.3	0.0031	0.0006	—
Total PUFA	4.2 ± 0.4	4.4 ± 0.2	0.9 ± 0.3	0.8 ± 0.1	0.0000	—	—

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

in the seawater medium was not affected by temperature, that in freshwater bacteria was significantly higher at 20 than 5°C (19.1 *vs.* 15.7%). A different effect was noted in the proportions of 16:0, in that temperature did not affect its level in bacteria grown in freshwater, but its proportion in seawater bacteria was significantly higher at 20°C (14.6%) than at 5°C (10.5%). Overall, the PE of *Vibrio* grown at 20°C in both seawater and freshwater media had a significantly higher proportion of saturated fatty acids than that of bacteria grown at 5°C. However, only at 5°C was there a significant difference between bacteria grown in seawater and freshwater in terms of the total level of saturated fatty acids in the PE.

The predominant isomer of 16:1 in PE was *cis* 16:1n-7 which accounted for 18–23% of the total PE fatty acids. Growth conditions had no major effect on the level of this fatty acid in PE although its level was slightly, but significantly, lower in freshwater than seawater at 5°C. The only *trans*-monoenoic fatty acid detected was *trans* 16:1n-7 which comprised 12.8 and 15.4% of the total PE fatty acids in *Vibrio* grown at 5°C in seawater and freshwater medium, respectively. The level of this *trans*-monoenoic fatty acid was markedly lower in the corresponding cultures grown at 20°C, *i.e.,* 4.4 and 6.3% in seawater and freshwater, respectively. The proportions of 20:5n-3 in PE in bacteria grown at 5°C in seawater and freshwater were very similar at around 4.3%. Likewise there was no difference between bacteria grown in seawater and freshwater medium at 20°C in terms of the percentage of 20:5n-3 in PE but the percentage (around 0.9%) was significantly lower than were those of the 5°C cultures. As a consequence of temperature-related changes in the contents of monoenoic and polyunsaturated fatty acids, the PE of bacteria grown at 5°C was more unsaturated overall than that of bacteria grown at 20°C. As with total lipid, analysis of variance of the pooled mass for individual fatty acids showed that the effect of temperature was generally more significant than that of medium composition and that significant interaction between these two factors occurred with only a few fatty acids.

The overall fatty acid composition of PG (Table 4) was notably different from that of PE in that it contained higher proportions of unsaturated fatty acids. Conversely, the overall proportion of saturated fatty acids was lower in PG than PE. Bacteria grown in either freshwater or seawater medium at 20°C contained higher proportions of saturated fatty acids in this phospholipid than those cultured at 5°C.

The levels of monoenoic fatty acids in PG were significantly reduced by growth at 5°C regardless of whether the bacteria were cultured in freshwater or seawater media. Thus, for seawater medium, monoenoic fatty acids comprised 60.2 and 66.2% of total PG fatty acids at 5 and

TABLE 4

Effects of Growth Temperature and Medium Composition on Fatty Acid Composition (wt%) of Phosphatidylglycerol from *Vibrio* sp. Showing Significance of Each Factor and Their Interaction[a]

Fatty acids	5°C		20°C		Significance level *(P)*		
	Seawater	Freshwater	Seawater	Freshwater	Temp.	Medium	Interaction[b]
i-12:0	0.1 ± 0.1	0.2 ± 0.0	ND	ND	—	—	—
12:0	0.7 ± 0.6	0.9 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	—	—	—
i-13:0	0.5 ± 0.4	0.6 ± 0.1	0.2 ± 0.1	ND	—	—	—
14:0	1.5 ± 0.4	2.5 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.0000	0.0081	0.0029
14:1n-5	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	—	—	—
i-15:0	5.4 ± 0.8	6.1 ± 0.2	4.4 ± 0.3	5.7 ± 0.6	—	0.0154	—
a-15:0	0.4 ± 0.2	0.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	—	—	—
15:0	2.7 ± 0.3	1.6 ± 0.3	4.1 ± 0.3	4.0 ± 0.9	0.0001	0.0351	—
15:1n-6	1.5 ± 0.6	0.9 ± 0.1	1.9 ± 0.2	1.5 ± 0.3	0.0204	0.0316	—
16:0	10.3 ± 0.5	11.0 ± 0.6	13.0 ± 1.3	12.8 ± 0.7	0.0017	—	—
trans 16:1n-7	15.2 ± 1.0	15.1 ± 0.4	6.1 ± 3.6	8.5 ± 0.8	0.0007	—	—
16:1n-9	2.1 ± 0.2	1.9 ± 0.2	2.2 ± 0.2	2.6 ± 0.3	0.0053	—	0.0346
cis 16:1n-7	23.4 ± 1.1	26.9 ± 0.3	27.7 ± 3.8	25.2 ± 0.7	—	—	0.0165
i-17:0	0.4 ± 0.2	0.3 ± 0.1	1.3 ± 0.3	1.6 ± 0.5	0.0002	—	—
17:0	0.8 ± 0.1	0.3 ± 0.0	3.5 ± 0.6	2.7 ± 0.1	0.0000	0.0004	—
17:1n-8	8.2 ± 0.3	3.6 ± 0.3	16.0 ± 0.3	{ 16.8 ± 3.1	0.0000	0.0011	0.0047
17:1n-6	1.4 ± 0.2	0.3 ± 0.2	1.7 ± 0.3				
18:0	0.6 ± 0.1	0.3 ± 0.1	2.0 ± 0.2	1.3 ± 0.2	0.0000	0.0005	—
18:1n-9	2.0 ± 0.2	0.8 ± 0.5	4.7 ± 0.8	4.3 ± 0.8	0.0000	—	—
18:1n-7	5.6 ± 0.4	4.8 ± 0.6	5.3 ± 0.4	6.4 ± 0.7	—	—	0.0296
19:1n-8	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.1	—	—	0.0110
20:5n-3	16.2 ± 1.8	20.2 ± 0.6	3.7 ± 1.6	4.3 ± 0.3	0.0000	0.0499	—
Total sat.	23.4 ± 1.2	24.2 ± 0.7	30.1 ± 2.2	29.8 ± 0.5	0.0000	—	—
Total mono.	60.2 ± 2.7	55.0 ± 0.4	66.2 ± 0.6	65.9 ± 0.7	0.0000	0.0170	0.0345
Total PUFA	16.2 ± 1.8	20.2 ± 0.6	3.7 ± 1.6	4.3 ± 0.3	0.0000	0.0499	—
Unidentified	0.2 ± 0.2	0.6 ± 0.3	ND	ND			

[a]Values are means ± SD of three cultures.

[b]Interaction between temperature and medium composition. Mono., monoenoic fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected; sat., saturated fatty acids; —, not significant. *i-, iso; a-, anteiso.*

20°C respectively, with the corresponding values for freshwater medium being 55.0 and 65.9%. The only monounsaturated fatty acid which did not decrease or stayed constant in proportion between 20 and 5°C was *trans* 16:1n-7, which exhibited a marked increase in proportion at the lower growth temperature with both media. As with total lipid and PE, the only polyunsaturated fatty acid present in PG of the *Vibrio* was 20:5n-3 which accounted for 16.2% of the total fatty acids present in PG of bacteria grown at 5°C in seawater and 20.2% in the same phospholipid of bacteria grown at the same temperature in freshwater medium. The proportion of 20:5n-3 was very notably and significantly ($P < 0.001$) lower in the PG from bacteria grown in either medium at 20°C. As with total lipid and PE, the fatty acid composition of PG was apparently more influenced by the temperature at which the *Vibrio* was grown than whether seawater or freshwater medium was employed. The significant interactions between the effects of temperatures and medium composition on fatty acid composition occurred mainly with monoenoic fatty acids.

DISCUSSION

The lipid class composition of the *Vibrio* species examined in the present study is relatively simple with PE and PG predominating and is typical of Gram-negative bacteria in general (1). However, the bacterium apparently lacks the phosphatidylserine and cardiolipin which have been reported for psychrophilic *Vibrio* isolated from marine sediments (9).

The bacterium possessed two types of fatty acids which are not typical of bacteria in general, namely, polyunsaturated fatty acids and *trans*-monoenoic fatty acids. Although these fatty acids have been reported as occurring independently in *Vibrio* (2,10,11,26), as far as we are aware this is the first report of their simultaneous presence in the lipids of a single bacterial species. Since the nutrient was defatted before its incorporation into the growth media, all the fatty acids extracted from the bacteria must have been synthesized *de novo* and not taken up preformed from the medium. The level of 20:5n-3 in the total lipid of the *Vibrio* examined here, however, is considerably less than that observed for bacteria isolated from deep-sea sources (2) where this polyunsaturated acid can account for up to 36.7% of the total fatty acids. As with *Vibrio* species in general (9,17), 16:1 was a major fatty acid of the *Vibrio* species examined in the present study. However, the levels of *iso*-13:0 and *iso*-15:0, particularly the latter, were much higher than have previously been reported for *Vibrio* and other marine species of bacteria (2,9,17,27). It is notable that the reported fatty acid compositions of *Vibrio* bacteria do vary widely (2,9).

Despite their overall rarity in bacteria, *trans* fatty acids have been found in some strains of marine bacteria including *Pseudomonas atlantica* (28) and an unidentified *Vibrio* sp. grown in seawater (11). The levels of *trans*

16:1n-7 in both the PG and PE of the *Vibrio* employed in this study were notably higher, especially at 5°C, than those observed by Okuyama *et al.* (10) in another *Vibrio* species. Furthermore, in the *Vibrio* studied here, the proportions of the *trans*-monoenoic fatty acid in PE and PG were similar, whereas in the bacteria studied by Okuyama *et al.* (10) the *trans* 16:1n-7 was present in higher concentration in PE than PG. The biological significance of *trans* fatty acids in bacteria remains to be established.

Given the relatively high levels of 18:1n-9 present in the lipids of the *Vibrio*, it seems likely that, in addition to the enzymes of the so-called anaerobic pathway of desaturation (1), the bacterium also possesses the aerobic Δ9 desaturase of eukaryotic cells. The wide spectrum of monounsaturated fatty acids present also indicates that the inferred Δ9 desaturase can act on a wide range of both odd- and even-numbered saturated fatty acids and that the *Vibrio* contains an active system for the elongation of acyl chains. Although the profile of monounsaturated fatty acids can be explained by the existence in the *Vibrio* of a single desaturase, the Δ9 desaturase, the synthesis of 20:5n-3 requires the bacterium to possess Δ12, Δ15, Δ6 and Δ5 desaturases if its formation proceeds *via* conventional pathways found in eukaryotes. It is notable that intermediates between monounsaturated and the pentaenoic fatty acid product are not found, even in trace amounts, in the *Vibrio* lipid. Although uncommon, this situation is similar to that found with the dinoflagellate *Crypthecodinium cohnii* in which 22:6n-3 is a major fatty acid and no other polyunsaturated fatty acid comprises more than 0.1% of the total fatty acids (29). It remains to be established whether the desaturations leading to the formation of 20:5n-3 in the *Vibrio* involve acyl carrier protein derivatives or fatty acids esterified in phospholipids, as substrates.

The effects of temperatures on lipid composition were far more pronounced than those of the salinity of the medium. The increased proportions of PE in lipid at low growth temperature is in keeping with the situation observed with poikilothermic animals (7). It is notable, however, that the increase in the proportion of PE was balanced by reductions in those of NEFA rather than those of PG. This lack of change in the proportion of PG is consistent with the fact that significant alterations in the level of this phospholipid in the halophilic *Vibrio costicola* occur only when the salt concentration of the medium is increased to values far in excess of those employed here (30).

Temperature effects on *Vibrio* fatty acid compositions have been reported previously with the actual effect varying with species (9). The present study showed a definite increase in the proportion of polyunsaturated fatty acids in the bacterial lipid as temperature decreased. This is in keeping with the situation reported for many poikilothermic vertebrate and invertebrate animals (7). The increase in the level of 20:5n-3 in the *Vibrio* by lowering growth temperature from 20 to 5°C was slightly less than the sixfold increase in the proportion of 22:6n-3 which occurs when *Vibrio marinus* is subjected to a decrease in environmental temperature from 20 to 2°C (2).

Also very notable was the increase in the content of *trans* fatty acids as the growth temperature decreased. This is in marked contrast to the report of Okuyama *et al.* (10) who found that the *trans*-monoenoic fatty acid content of the lipid from *Vibrio* sp. strain ABE-1 was higher at 20 than 5°C. Previously, increases in the proportion of *trans* 16:1n-7 with increasing growth temperature have been explained in terms of the *trans* fatty acid increasing the phase transition temperature of the membrane phospholipid as an adaptation to changes in the ambient temperature (10). However, this explanation is not applicable to the situation observed in the present study where the level of *trans* 16:1n-7 was higher at 5 than 20°C. Since the proportions of *trans* 16:1n-7 and 20:5n-3 both increased with decreasing temperature, it is possible that the net effect is a cooperative response between two fatty acids in the same phospholipid molecule which ensures molecular packing compatible with membrane bilayer properties required at lower temperature.

In summary, the present study demonstrates that 20:5n-3 and *trans* 16:1n-7 occur simultaneously in both PG and PE of a *Vibrio* species of bacterium and that a reduction in growth temperature significantly increases the proportions of both fatty acids in these lipids. Studies are continuing with the *Vibrio* to establish the pathways of 20:5n-3 biosynthesis. In addition, the positional distribution of *trans* 16:1n-7 and 20:5n-3 in PG and PE and how this is influenced by growth temperature, is under examination.

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2-Hydroxyhexadecanoic and 8,9,13-Trihydroxydocosanoic Acid Accumulation by Yeasts Treated with Fumonisin B_1

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Fumonisin B_1 is a sphingolipid-like compound that enhances the accumulation of yeast sphingolipids and 2-hydroxy fatty acids. These lipids occur both as freely extractable and cell bound components in yeast fermentations. Both free and bound 2-hydroxy fatty acids produced by *Pichia sydowiorum* NRRL Y-7130 were increased when fumonisin B_1 (50 mg/L) was added to the usual growth medium containing yeast extract/malt extract/peptone/glucose. Fumonisin-treated cultures contained 38 mg/L more 2-hydroxyhexadecanoic and 15 mg/L more 2-hydroxyoctadecanoic acids than did untreated cultures. By contrast, fumonisin inhibited the accumulation of free 8,9,13-trihydroxydocosanoic acid in *Rhodotorula* sp. YB-2501 cultures, leading to 240 mg/L lower trihydroxy acid production than by untreated cultures.
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Water-soluble fumonisin B_1, a propane-1,2,3-tricarboxylic acid 14,15-diester of the 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane base (1), is a sphingolipid-like compound (Fig. 1). It has been shown to cause, in the presence of [^{14}C]serine, labeled sphinganine (D-*erythro*-2-amino-1,3-octadecanediol) accumulation in rat hepatocytes (2). Fumonisin B_1 is a cerebral toxin (3) and phytotoxin (4) produced by the mold *Fusarium moniliforme.* However, the effect of fumonisin on microorganisms and their lipid composition has not yet been determined.

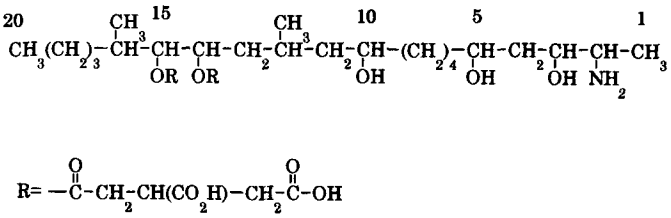

FIG. 1. Structure of fumonisin B_1.

The yeast *Pichia (Hansenula) ciferri* NRRL Y-1031 is known for both its extracellular production of triacetylsphinganine and tetracetyl-4-oxysphinganine (5) and its ability to synthesize sphingolipid bases (6,7). When *P. ciferri* Y-1031 is treated with fumonisin, accumulation of sphinganine, 4-D-hydroxysphinganine and other polar lipids (8) is increased in both the free and bound lipid fractions.

As microbial cultures produce sphingolipids and polar hydroxy fatty acids in freely extractable and bound glycolipid form (5,9,10), specific yeast strains that produce either a 2-hydroxy (11) or 8,9,13-trihydroxy (12) fatty acid extracellularly were studied to assess the effects of fumonisin. In order to detail the lipid changes that occur in fumonisin-treated cultures, both free and bound forms of the hydroxy acids were determined.

P. sydowiorum NRRL Y-7130 produces aerobically an extracellular 2-D-hydroxyhexadecanoic acid in growth-limiting glucose/malt extract medium (11). *Rhodotorula* sp. YB-2501 produces extracellular *erythro*-8,9,13-triacetoxydocosanoic (12) acid under similar restricted growth conditions. For direct comparison of yeast hydroxy acids with sphingolipid (8) accumulation, culture broths were separated by centrifugation into supernatant and particulate (cell/particle) fractions. Each fraction contained freely extractable lipids. From the defatted cells, a bound lipid fraction was extracted after saponification (8,9). The effect of fumonisin B_1 on yeast hydroxy fatty acid production is discussed in respect to the free and bound lipid composition determined.

MATERIALS AND METHODS

Materials and biologicals. 2-DL-Hydroxypalmitic and 2-DL-hydroxystearic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Standard 2-D-hydroxystearic, 3-D-hydroxypalmitic and *erythro*-8,9,13-trihydroxydocosanoic acids were obtained from the NRRL chemical collection of Stodola and Vesonder (5,11,12). Fumonisin B_1 was produced by a *Fusarium moniliforme* fermentation of corn (13) and purified by XAD-2 anion exchange column chromatography in sequence with high-performance liquid chromatography (HPLC).

P. sydowiorum Y-7130 and *Rhodotorula* sp. YB-2501, obtained from the ARS Culture Collection (Peoria, IL), were maintained on 1.6% (wt/vol) agar slants containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 2.7% glucose medium (11) adjusted to pH 6.5. Growth of the yeasts in liquid medium (g wet wt/L) was compared with aerobic cultures grown on 1% (wt/vol) glucose and 5% malt extract medium (12). Because the latter medium increased extracellular accumulation of both 2-hydroxy (11) and trihydroxy (12) fatty acids, but lowered cell yields, the two different media served as useful monitors to relate cellular growth to accumulated fatty products. When yeast cells were dried at 70°C to constant weight, a mean value of 0.28 g dry wt was obtained per g wet weight.

Media (100 mL medium per 300-mL Erlenmeyer flask) containing either 5 mg or no (control) exogenous fumonisin B_1 were inoculated with 3-d cultures (1 mL) of fully grown yeasts. After 5-7 d of aerobic (180 rpm, rotary shaker) incubation at 25°C, fermentation broths were centrifuged.

Lipid and fatty acid extraction. Extracellular fatty acids were extracted as described (11,12). After centrifugation, supernatants were acidified to pH 3 and extracted (Fraction 1). *Pichia* Y-7130 supernatant was extracted with diethyl ether (3 × 50 mL), and *Rhodotorula* YB-2501 supernatant was extracted with ethyl acetate (2 × 50 mL).

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Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; TLC, thin-layer chromatography.

Centrifuged pellets containing cells and lipid particles were extracted by a modified Folch procedure (14,15) using two 15-mL portions of warm chloroform/methanol (2:1, vol/vol) (Fraction 2). The respective extracts were combined and concentrated to dryness with a rotary evaporator. Fraction 2 lipids were resuspended in 8 mL chloroform/methanol (2:1, vol/vol), washed with water (0.5 mL) and dried over anhydrous Na_2SO_4. The drying agent was removed by decanting and the solvents were removed *in vacuo*.

Lipids bound to defatted cells (8–10), most likely glycolipids (5,16,17), were saponified with excess 1 N KOH (70% methanol; vol/vol) for 2 h at 70°C. The reaction mixture was acidified with H_2SO_4, mixed with 1 vol of chloroform and filtered through 5 g Celite. The Celite was extracted twice with 50-mL portions of chloroform/methanol (2:1, vol/vol), and the combined extracts were concentrated to dryness (Fraction 3). The yield of each fraction was determined by weighing.

Ester preparation. Lipids in Fractions 1–3 were not analyzed specifically for triacylglycerols, phospholipids and glycolipids, as has been done in other studies (18–22). Instead, a portion of each fraction (5 mg) was saponified with excess 0.5 N KOH (1 mL) in aqueous ethanol (6% water, vol/vol) (12) for 30 min at 70°C. Hydrolysates were then cooled, acidified and extracted with warm chloroform/methanol. Each dried sample was suspended in a few drops of methanol, and the fatty acids were esterified with diazomethane.

Silicic acid chromatography. The methyl esters were separated over a Unisil (100–200 mesh; Clarkson Chemical Co., Williamsport, PA) silicic acid column using 3 g of silicic acid for up to 60 mg of lipids. The 2-hydroxy fatty esters of strain Y-7130 were separated by sequential elution (8 column volumes for each solvent): (i) 20% chloroform in petroleum ether (vol/vol); (ii) 100% chloroform; and (iii) 20% methanol in chloroform. The more polar trihydroxy fatty esters were eluted with (i) chloroform, and solvent mixtures containing (ii) 5%, and (iii) 30% methanol. Selected samples were also analyzed by thin-layer chromatography (TLC). Fractions were separated on silica gel G plates using toluene/dioxane/acetic acid (79:14:17, by vol) as solvent system. The fractions were made visible by acid-charring (23). Authentic methyl esters had the following R_f values: 2-hydroxyhexadecanoate (0.80), 3-hydroxyhexadecanoate (0.67), 2-hydroxyoctadecanoate (0.80), 9,10-dihydroxyoctadecanoate (0.53) and 8,9,13-trihydroxydocosanoate (0.30).

Gas chromatography (GC) and mass spectrometry (MS). Methyl esters of 2-hydroxy fatty acids were dissolved in chloroform/methanol and analyzed immediately by capillary GC. The 2-hydroxy esters were separated isothermally at 230°C using a 15-m SE-30 column (SPB-1; Supelco Inc., Bellefonte, PA) and were quantified by calculating peak areas relative to authentic standards. Authentic 2-DL-hydroxypalmitate and 2-DL-hydroxystearate gave mean values of 36.2 and 33.3×10^6 unit areas per mg, respectively, using a Hewlett-Packard 5890 instrument (Avondale, PA). The less-volatile trihydroxy fatty ester was analyzed at 270°C as trimethylsilyl derivative that was generated with Tri-Sil TBT reagent (Pierce Chemical Co., Rockford, IL). Authentic methyl 8,9,13-tri(trimethylsiloxy)-docosanoate gave a mean peak area of 57.7×10^6 units per mg of free acid.

Structures were confirmed by GC/MS using a fused silica, 25-m capillary column coated with methyl silicone in tandem with a Hewlett-Packard 5970 Mass Selective Detector (70 eV; Palo Alto, CA). Methyl esters were separated on the GC column by temperature programming from 160–250°C at an 8°C increase per min and holding at 250°C for 10 min. Authentic methyl 2-DL-hydroxyhexadecanoate (molecular weight 286) gave characteristic molecular ion and mass ion fragments of *m/z* 227, 208, 182, 159, 127, 97 and 83. Authentic methyl 8,9,13-tri(trimethylsiloxy)docosanoate (molecular weight) 618) gave characteristic ions of *m/z* 401, 373, 318, 283, 245, 229, 147 and 129. The mass spectra of these compounds have been characterized earlier by others (24,25).

RESULTS

Fumonisin B_1 did not significantly affect the cell yield of the yeasts. *Rhodotorula* sp. YB-2501 cultures produced 33 g wet wt/L of cells when grown on yeast extract/malt extract/peptone/glucose medium containing either 50 mg/L or no fumonisin B_1. In the same medium, growth of fumonisin-treated *P. sydowiorum* Y-7130 was retarded slightly by 10%, yielding 41 g wet wt/L without (control) and 36 g with fumonisin treatment. With the restrictive growth medium of glucose/malt extract, cell yields averaged 8 g wet wt/L for strain YB-2501 and 21 g/L for strain Y-7130, regardless of fumonisin treatment. Thus cellular yields were affected more by the nutrients than by fumonisin.

Fumonisin effect on 2-hydroxy fatty acid accumulation. With *P. sydowiorum* Y-7130, which is known to produce extracellular accumulation of 2-D-hydroxyhexadecanoic (hydroxy C_{16}) and 2-hydroxyoctadecanoic (hydroxy C_{18}) acids (11), fumonisin B_1 supplementation was found to increase 2-hydroxy fatty acid production. Yeast hydroxy fatty acids are extractable in both free and bound forms and accumulate more readily in a glucose/malt extract (11, 12) rather than in yeast extract/malt extract/peptone/glucose fermentation broths. The data in Table 1 are therefore presented separately to allow detailed assessment. Cellular 2-hydroxy fatty acid accumulation was significantly affected by fumonisin in vigorously growing cultures using a yeast extract/malt extract/peptone glucose medium. However, when grown in the restrictive glucose/malt extract medium, the fumonisin effect on 2-hydroxy acid accumulation was less noticeable.

In yeast extract/malt extract/peptone/glucose medium, fumonisin-treated strain Y-7130 cultures accumulated 38 mg/L more free 2-hydroxy C_{16} and 15 mg/L more free 2-hydroxy C_{18} acids (Table 1; combining Fractions 1 and 2) than did untreated control cultures. The fumonisin-treated cultures accumulated increasing amounts of hydroxy C_{18} (14% increased to 19% of total peak areas by GC) rather than hydroxy C_{16} (75% decreased to 50%) esters. Bound hydroxy C_{16} and C_{18} acids in Fraction 3 (total of 24 mg/L) also increased in fumonisin-treated cells. In addition, 2-hydroxy fatty acids bound to fumonisin-treated cells appeared to be a larger portion (2.4/25) of the total, bound polar lipids than those from untreated cells (0.04/44).

Large amounts of cell bound lipids (0.3 to 1.1 g/L) were recovered in saponified Fraction 3. Fumonisin-treatment caused a decrease in bound, nonpolar lipids (1.1 g

TABLE 1

Nonpolar Lipids, Polar Lipids and 2-Hydroxy Fatty Acids Recovered from *Pichia sydowiorum* Y-7130 Treated with Fumonisin B_1[a]

Extracted fraction[b]	Growth medium	Fumonisin added (mg/L)	Recovered lipid[c] (mg/100 mL culture)		Recovered 2-hydroxy fatty acids[d] (mg/100 mL culture)	
			Nonpolar	Polar	C_{16}	C_{18}
Supernatant	YMP	0	1.8	4.4	0.01	0.01
(1)	YMP	50	2.0	5.6	0.07	0.01
	GM	0	1.8	6.5	2.2	0.1
	GM	50	1.4	8.6	2.8	0.4
Cell/particle	YMP	0	2.7	18.0	0.1	0.00
(2)	YMP	50	2.1	23.0	3.8	1.5
	GM	0	2.6	28.0	19.9	1.4
	GM	50	2.4	35.0	14.9	3.8
Bound	YMP	0	110	44.0	0.04	0.00
(3)	YMP	50	72.0	25.0	1.7	0.7
	GM	0	27.0	33.0	9.7	0.6
	GM	50	19.0	44.0	14.1	2.0

[a] Abbreviations: C_{16}, methyl 2-hydroxyhexadecanoate; C_{18}, methyl 2-hydroxyoctadecanoate; GM, growth medium of glucose/malt extract; nonpolar, methyl ester of nonpolar lipid fraction recovered from silicic acid column; polar, methyl ester of polar lipid fraction recovered from silicic acid column; YMP, growth medium of yeast extract/malt extract/peptone/glucose.

[b] Liquid cultures of the yeast were separated by centrifugation. Supernatant, cell/particle and saponified residue (bound) fractions were subsequently extracted with solvents.

[c] Lipid fractions were saponified and methyl esters prepared before partition by silicic acid chromatography. Nonpolar lipids were eluted with 20% chloroform/petroleum ether (vol/vol), and polar lipids were eluted with chloroform.

[d] Determined by capillary gas chromatographic peak areas relative to standard C_{16} and C_{18} compounds, 36.2 and 33.3 $\times$ 10^6 unit area per mg free acid, respectively.

decreased to 0.7 g/L), whereas polar lipids were not affected (0.4 g to 0.3 g/L).

TLC analysis of the methylated polar lipids showed four prominent fractions at R_f 0.65, 0.75, 0.95 and 1.0, one of which was similar to authentic 2-hydroxy C_{16}–C_{18} methyl esters (R_f 0.80 $\pm$ 0.05). Analyses in combination with GC indicated that the 2-hydroxy acids of fumonisin-treated cells were a significant portion of the total, bound polar lipids (16.1/44 or 37%). GC/MS of selected samples confirmed the presence of 2-hydroxy C_{15}–C_{18} methyl ester homologues (23,24). In addition to the expected molecular ions, fragment ions $[M-59]^+$ were most prominent. For example, authentic methyl 2-hydroxyhexadecanoate and samples from the yeast fractions (Table 1) gave identical retention times and MS fragment ions.

Decrease in 8,9,13-trihydroxydocosanoic acid accumulation. Table 2 contains data for *Rhodotorula* sp. YB-2501 which is known to produce extracellular, freely extractable *erythro*-8,9,13-triacetoxydocosanoic acid (12). In contrast to strain Y-7130 (Table 1), the fumonisin-treated strain YB-2501 accumulated approximately 240 mg/L less free trihydroxy acid in Fraction 2 than did the untreated controls (Table 2). Also, fumonisin B_1 was found to inhibit free trihydroxy fatty acid formation (combined Fractions 1 and 2) in both media. Because both free nonpolar and polar lipids decreased in fumonisin-treated cultures, fumonisin appears to nonspecifically inhibit accumulation of free cellular lipids of the strain YB-2501. However, bound trihydroxy acid of cells grown on yeast extract/malt extract/peptone/glucose medium was not appreciably affected by fumonisin (0.27 g/L for treated culture and 0.24 g/L for control). Thus, the trihydroxy acid represented a major portion of the free polar (25/26) rather than the bound polar (1.9/24) lipids.

The trihydroxy fatty ester was characterized by TLC (R_f 0.30), and the structure was confirmed by GC/MS as that of a 8,9,13-trihydroxydocosanoic acid.

DISCUSSION

Fumonisin B_1 in animal cells and tissues (2,26) inhibits a cellular ceramide synthase; whereby, sphinganine accumulates while sphingenine synthesis is inhibited. Unlike animal cells, *P. ciferri* Y-1031 produce extracellular tetraacetyl-4-oxysphinganine and triacetylsphinganine (5) and may synthesize free sphinglipid bases by an alternate pathway (6,7). Because fumonisin B_1 enhances the accumulation of unknown polar lipids as well as sphingolipids (8) in strain Y-1031 cultures, polar lipid changes need to be considered when other yeast strains are treated with fumonisin (Table 1).

In our studies, both 2-hydroxy fatty acids of *P. sydowiorum* H-7130 (Table 1) and sphingolipids of strain Y-1031 accumulated in fumonisin-treated cultures. Fumonisin B_1 was not particularly toxic to the yeast cells as judged by cell yields. As expected, fumonisin increased both types of polar lipids when growth occurred in a restrictive glucose/malt extract medium. Nonpolar lipids bound in cells (Fraction 3) decreased significantly from 1.1 g/L to 0.72 g/L upon fumonisin treatment. The

TABLE 2

Nonpolar Lipids, Polar Lipids and 8,9,13-Trihydroxydocosanoic Acid Recovered from *Rhodotorula* sp. YB-2501 Treated with Fumonisin B_1[a]

Culture fraction[b]	Growth medium	Fumonisin added (mg/L)	Recovered lipid[c] (mg/100 mL culture)		Recovered trihydroxy fatty acid[d] (mg/100 mL culture)
			Nonpolar	Polar	C_{22}
Supernatant	YMP	0	1.9	11.0	3.1
(1)	YMP	50	1.0	13.0	1.6
	GM	0	2.7	11.0	1.4
	GM	50	1.1	4.5	0.1
Cell/particle	YMP	0	11.0	26.0	25.0
(2)	YMP	50	0.5	3.6	0.7
	GM	0	5.6	30.0	12.0
	GM	50	2.4	7.0	1.7
Bound	YMP	0	13.0	24.0	1.9
(3)	YMP	50	13.0	27.0	2.5
	GM	0	6.6	12.0	0.3
	GM	50	2.5	4.8	0.2

[a] Abbreviations: C_{22}, trimethylsilylated derivative of methyl trihydroxydocosanoate; GM, growth medium of glucose/malt extract; nonpolar, methyl ester of nonpolar lipid fraction from silicic acid column; polar, methyl ester of polar lipid fraction recovered from silicic acid column; YMP, growth medium of yeast extract/malt extract/peptone/glucose.

[b] Liquid cultures of the yeast were separated by centrifugation. Supernatant, cell/particle and saponified residue (bound) fractions were subsequently extracted.

[c] Lipid fractions were saponified and methyl esters prepared before partition over silicic acid column. Nonpolar lipids were eluted with chloroform, and polar lipids were eluted with 5% methanol/chloroform (vol/vol).

[d] Trimethylsilylated C_{22} determined by capillary gas chromatographic peak areas relative to standard 8,9,13-trihydroxydocosanoate (57.7×10^6 unit area per mg free acid).

lipid changes caused by exogenous fumonisin were similar in respect to 2-hydroxy acids and sphingolipids. Whether other bound polar lipids (Fraction 3) functioned interchangeably with either 2-hydroxy fatty acids or sphingolipids was not determined. However, fumonisin appeared to stimulate the release and turnover of lipids bound to glycolipids and glycosphingolipids (5,17).

The free and bound hydroxy fatty acids of *P. sydowiorum* Y-7130 were found to be mainly 2-hydroxy C_{15}–C_{18} acids, suggesting a prominent C-2 rather than a C-3 hydroxylating system. Thus, analogous to the 2-hydroxy C_{20}–C_{25} fatty acid synthesis by Caribbean sponges (27), a 2-oxidation system involving 2-peroxylactone intermediates (28,29) may be expressed by strain Y-7130.

In contrast, fumonisin B_1 inhibited free 8,9,13-trihydroxydocosanoic acid accumulation (combined Fractions 1 and 2). Moreover, the trihydroxy acid in the bound form (Fraction 3) was not affected by fumonisin. This implies that fumonisin B_1 affects accumulation of trihydroxy fatty acid in yeast in a different way than either 2-hydroxy acid (Table 1) or sphingolipids (8).

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Effect of Cell Density on Cytotoxicity of Ether Lipid Analogues in Variants of B16 Murine Melanoma

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Ether lipids are defined here as analogues of naturally occurring lysophosphatidylcholines with cytotoxic activity against neoplastic cells. The activity of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET18OMe) and 3-hexadecylmercapto-2-methoxymethyl-propyl-1-phosphocholine (Ilmofosine®) (BM 41.440) was tested in variants of B16 murine melanoma, grown in adhesion cultures (B16F1 with low metastatic potential; B16F10 and B16BL6 with high metastatic potential). Cytotoxicity was evaluated by counting the cells that survived after 24 h of drug exposure. Cholesterol, sphingomyelin, total phospholipid and phosphatidylcholine levels were determined. After 24 h of drug exposure, cultures of the B16BL6 variant contained a larger number of cells, especially when high drug concentrations (100–250 μM) were used, than cultures of the B16F1 and B16F10 variants. The sensitivity to ET18OMe of the three variants was evaluated at different cell densities (at each density the dose was equalized per number of cells/well; 0.1 μmol/10^6 cells/well). In B16F1 and B16F10 cultures the dose-response curve was not affected by the number of cells/well, while in B16BL6 no more than 20% of the cells were killed at all cell densities measured. A linear relationship was noted between cell density and cholesterol/phospholipid and sphingomyelin/phosphatidylcholine ratios in the resistant variant B16BL6, confirming that lipid composition modulates the cytotoxic activity of ether lipids.
Lipids 28, 403–406 (1993).

Ether lipids (EL) constitute a class of biological response modifiers that are cytotoxic to neoplastic cells *in vitro* (1) and protect animals from proliferative metastasis by a number of primary tumors (2). Their mechanism of action and the reasons for their selective action are still to be clarified. EL do not affect DNA metabolism (3), but affect plasma membrane biochemical and biophysical properties (1,4–7).

Two main biochemical parameters have been recognized to affect EL cytotoxic activity, namely membrane cholesterol (CHOL) and endogenous EL levels. CHOL affects the susceptibility of tumor cells to EL either when incorporated into neoplastic cells in culture (8,9), or when variants of leukemic cells rich in CHOL were exposed to EL (10), or when cell membrane cholesterol was depleted by incubating the cells with AL 721 (a mixture of phospholipids, PL) (11). As the metabolism of natural ether lipids appears to be impaired in some tumors (12), EL metabolism may also be affected in these tumors. However, this has not yet been fully clarified (13,14).

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Abbreviations: BM 41.440, 3-hexadecylmercapto-2-methoxymethyl-propyl-1-phosphocholine (Ilmofosine®); CHOL, cholesterol; EDTA, ethylenediaminetetraacetic acid; EL, ether lipids; ET18OMe, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; FBS, fetal bovine serum; i.v., intravenous; PC, phosphatidylcholine; PL, phospholipids; s.c., subcutaneous; SPH, sphingomyelin.

Few data are available in regard to the cytotoxic activity of EL on cells grown in adhesion; however, melanomas seem less sensitive to EL than are other tumors (15). In the present study, the cytotoxic activity of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET18OMe) and 3-hexadecylmercapto-2-methoxymethyl-propyl-1-phosphocholine (BM 41.440, Ilmofosine) was tested on three variants of the B16 murine melanoma cell line with different metastatic capacity cultured in adhesion.

MATERIALS AND METHODS

ET18OMe was provided by Dr. R. Nordstrom (Medmark Pharma, Munich, Germany) (16). BM 41.440 was provided by Boehringer (Mannheim, Germany) (17). Medium and culture flasks and wells were supplied by Flow Laboratories (Irvine, United Kingdom). Sodium pyruvate, glutamine, nonessential amino acids, vitamins, antibiotics, fetal bovine serum (FBS) and fungizone were obtained from Gibco (Parsley, United Kingdom). All reagents were of analytical grade.

F1, F10 and BL6 variants of the B16 murine melanoma cell line were supplied by Dr. J. Fidler (NCI, Frederick, MD). The metastatic capacity of the three clones as shown by Talmadge and Fidler (18) and tested at semiconfluence was as follows: B16F1, parental B16 melanoma, metastasizes poorly when tumors are implanted intravenous (i.v.) (experimental metastasis) or subcutaneous (s.c.) (spontaneous metastasis); B16F10 is highly metastatic after i.v. injection; B16BL6 is highly metastatic after i.v. and s.c. administration.

Cells were stored at −180°C in flasks containing 2×10^6 cells. Cells for experiments were taken from the frozen stock and used for not more than three passages. Cultures were shown to be mycoplasm-free.

Cells were grown in Eagle's minimal essential medium (with 2 g/L sodium bicarbonate and 20% FBS), supplemented with penicillin (0.02 IU/mL), streptomycin (0.1 mg/mL) and fungizone (0.5 mg/mL) and containing vitamins, nonessential amino acids, 1 mM sodium pyruvate and 4 mM glutamine, in a humidified atmosphere containing 5% CO_2 at 37°C. After five to six days, cells were detached by incubating at 37°C for 1 min with 0.25% trypsin in phosphate buffered saline containing 2 mM ethylenediaminetetraacetic acid (EDTA), and counted with a Coulter Counter (Coulter Electronics Ltd., Hapenden, Herts, United Kingdom) and a cell analyzer (Coulter Channalyzer C-1000).

Approximately 50,000 cells, in triplicate, were placed in 9-cm^2 wells with 2 mL of medium. When the cell number reached $40–50 \times 10^3/cm^2$, the medium was replaced with media containing different concentrations of ET18OMe or BM 41.440, and the cells were incubated for 24 h. To estimate cell density at the beginning of an experiment, additional control wells, also in triplicate, were run in parallel; cells were detached as described above at the time when drugs were added to the experimental groups, and counted.

Stock solutions containing 50–100 mg/mL ET18OMe or BM 41.440 in ethanol were prepared and stored in small aliquots. Immediately before each experiment, stock solutions were diluted with the medium used for growing cells to obtain final concentrations of 20, 40, 100, 150 and 250 μM. Final ethanol content was less than 0.1%. Control media contained similar concentrations of ethanol.

To study the relationship between cytotoxicity and cell density, treatment was started using cultures with various cell densities (25 to 250 × 10^3 cells/cm^2). The dose was adjusted according to the number of cells in the well at the beginning of the treatment (ET18OMe concentration was 0.1 μmol/10^6 cells/well).

Cytotoxic activity in cells detached as described above was measured by erythrosin B dye exclusion. Cytotoxicity test results were expressed as a percentage of viable cells (*i.e.*, cells that retained a permeability barrier against erythrosin B) after 24 h of incubation with EL *vs.* viable cells after 24 h incubation without EL.

For lipid analysis, 0.3 × 10^6 cells were seeded and grown at different densities in T75 cm^2 flasks for 48 to 72 h. Cells were detached as described above and counted, then washed, resuspended in 5 mL of 0.05% hydroquinone-containing methanol and sonicated. Cell lipids were extracted according to Folch *et al.* (19). Aliquots of the resulting chloroform phases were used to measure total CHOL, using the Lieberman-Burchard reaction and, after acidic digestion, Pi in total PL (20). Sphingomyelin (SPH) and phosphatidylcholine (PC) were separated by thin-layer chromatography (21) and their Pi content measured as described above.

Duncan's new multiple range test for multiple comparisons was used for statistical analysis.

RESULTS

B16 murine melanoma variants with different metastatic capacity, grown in adhesion cultures to reach approximately 40 × 10^3 cells/cm^2, were exposed for 24 h to a range of concentrations of ET18OMe and BM 41.440. Toxicity was dose-related in all variants, though more BL6 cells survived than in the other variants, after concentrations of 100 μM or more of ET18OMe (Fig. 1, panel A) and BM 41.440 (Fig. 1, panel B). For both drugs the approximate ID_{50} concentrations were 85 μM in F1, 106 μM in F10 and 150 μM in BL6.

In various neoplastic cells the cytotoxic effect of EL can be modulated by other lipids (8–11). In order to test whether the lower sensitivity of the BL6 variant was related to some intrinsic difference in lipid composition, CHOL and PL were measured in the three variants. CHOL and PL levels of murine melanoma variants were affected differently by cell density: PL tended to decrease in all variants with increasing cell number, whereas CHOL decreased only in F1 and F10, while in BL6 it was low at low cell density and rose gradually with cell number (Table 1).

SPH and PC levels were also measured under these conditions: as with CHOL, SPH levels dropped as cell density increased in F1 and F10, but increased slightly in BL6; PC remained unchanged. Thus SPH/PC ratios fell with increasing cell density in F1 and F10 (Table 2) ($P < 0.05$ in low compared to high density), while the ratio increased with cell density in BL6. SPH/PC ratios of the

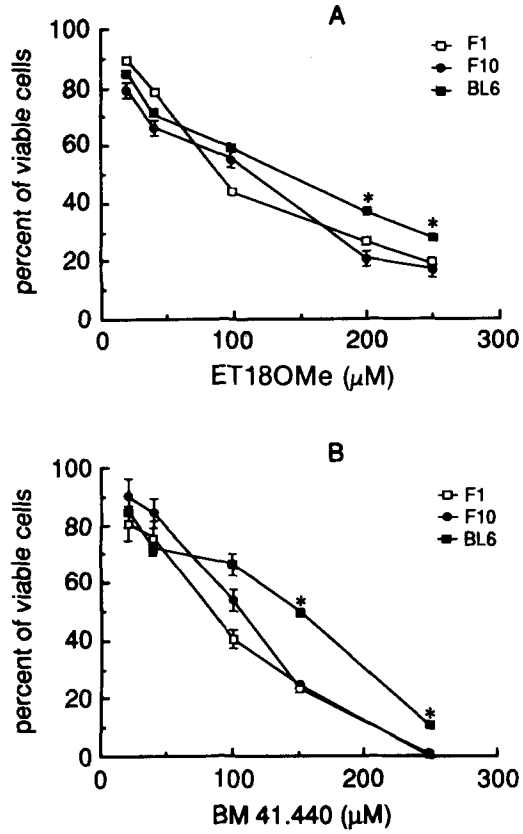

FIG. 1. Effect of 24-h exposure to ET18OMe (panel A) and BM 41.440 (panel B) on cytotoxicity in variants of B16 murine melanoma. 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol-3-phosphocholine (ET18OMe) and 3-hexadecyl-mercapto-2-methoxymethyl-propyl-1-phosphocholine (Ilmofosine®) (BM 41.440) were added when cell density was 50 × 10^3 cells/cm^2. Results are the percentages of viable cells after 24-h incubation with ether lipids (EL)/viable cells after 24-h incubation without EL. Each point is the mean ± SEM of 3–4 determinations. The asterisk designates $P < 0.01$ in comparison to F1 at similar drug concentration.

BL6 variant were significantly different ($P < 0.05$) from F1 at low density and from F1 and F10 at high density.

The data indicated that BL6 membranes differed in lipid content from those of the two other variants, especially when cultures were dense. This difference may have influenced the sensitivity to EL. The effect of cell density on the sensitivity of the cells to EL was therefore studied in all variants, using ET18OMe as the representative EL (Fig. 2). Cultures were grown for different time periods to obtain cell numbers per well ranging from 25 to 250 × 10^3 cells/cm^2 (densities at which lipid changes were observed), then ET18OMe was added. The doses were proportional to the number of cells/well (0.1 μmol/10^6 cells). In F1 and F10, cytotoxicity was dose related, rising with the number of cells in the well at the time of addition of the drug (for example, when cell density was 250 × 10^3 cells/cm^2 and the drug concentration 0.22 μmol/10^6 cells, only 10–20% of cells survived) (Fig. 2). In BL6, instead, as many as 70–80% of cells survived at all cell densities (and drug concentrations) tested, suggesting that a high percentage of BL6 cells was resistant to EL. This could be observed when cultures were dense and it correlated with high relative amounts of CHOL and SPH.

TABLE 1

Cholesterol (CHOL) and Phospholipid (PL) Content and CHOL/PL Ratio (in brackets) of B16 Murine Melanoma Variants in Relation to Cell Density (cell number/cm^2)

Cell density ($\times 10^3$/cm^2)	B16F1		B16F10		B16BL6	
	μg/10^6 cells ± SEM (CHOL/PL ratio ± SEM)					
	CHOL	PL	CHOL	PL	CHOL	PL
40–70	9.7 ± 0.5 (0.177 ± 0.007)	54.5 ± 1.6	11.9 ± 0.3 (0.227 ± 0.002)[a]	53.1 ± 4.4	6.5 ± 0.7[b] (0.121 ± 0.007)[a]	54.6 ± 5.6
71–100	8.7 ± 0.9 (0.197 ± 0.009)	44.9 ± 6.0	10.2 ± 0.9 (0.213 ± 0.016)	48.0 ± 2.3	9.9 ± 1.2[c] (0.211 ± 0.024)[c]	46.7 ± 1.4
101–130	7.2 ± 0.5 (0.195 ± 0.014)	37.2 ± 1.1	7.7 ± 0.2[c] (0.216 ± 0.017)	35.8 ± 2.4	8.4 ± 0.9[c] (0.212 ± 0.018)[c]	40.0 ± 1.9
131–170	6.5 ± 0.1[c] (0.204 ± 0.008)[c]	31.7 ± 0.9	5.7 ± 0.2[c] (0.187 ± 0.007)[c]	31.4 ± 1.1	9.3 ± 0.4[a,c] (0.251 ± 0.019)[a,c]	37.8 ± 1.7

[a]$P < 0.01$ in comparison to F1 and F10 at the same cell density.
[b]$P < 0.01$ in comparison to F1 at the same cell density.
[c]$P < 0.01$ in comparison to lowest cell density in the same variant.

TABLE 2

Sphingomyelin/Phosphatidylcholine (SPH/PC) Ratios of Melanoma Variants in Relation to Cell Density (cell number/cm^2)

Cell density ($\times 10^3$/cm^2)	SPH/PC ratio ± SEM		
	B16F1	B16F10	B16B16
40–70 $\times 10^3$	0.148 ± 0.025	0.105 ± 0.015	0.082 ± 0.07[a]
130–170 $\times 10^3$	0.075 ± 0.010[b]	0.061 ± 0.008[b]	0.113 ± 0.010[b,c]

[a]$P < 0.05$ in comparison to F1 at the same cell density.
[b]$P < 0.05$ in comparison to lowest cell density in the same variant.
[c]$P < 0.05$ in comparison to F1 and F10 at the same cell density.

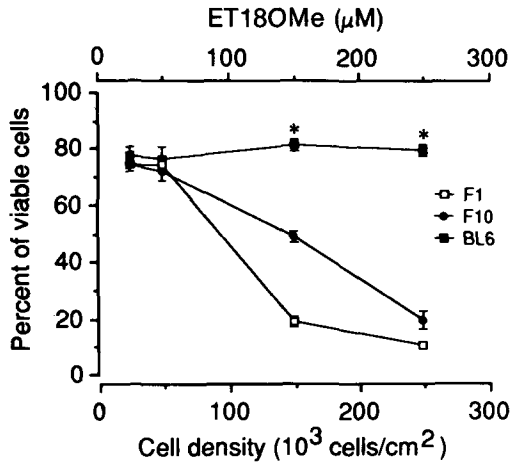

FIG. 2. Relationship between cell number and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol-3-phosphocholine (ET18OMe) cytotoxicity. The dose used (0.1 μmol/10^6 cells/well) was proportional to the number of cells before addition of the drug. Results are the percentages of viable cells after 24-h incubation with ether lipids (EL)/viable cells after 24-h incubation without EL. Each point is the mean ± SEM of 3–4 determinations. The asterisk designates $P < 0.01$ compared to B16F1 and B16F10.

DISCUSSION

The data confirm previous observations on the critical role of certain lipids on EL activity (8–11), but also demonstrate that endogenous conditions affecting lipid composition (in this case, cell density) can influence the effect of EL even within the same cell line. CHOL/PL and SPH/PC ratios are often taken as indices of membrane fluidity: the higher these ratios, the lower membrane fluidity (21). This could be an important factor since EL are known to exert their cytotoxic activity on the membrane. Blebs and holes have been observed in plasma membranes upon ET18OMe treatment of HL60 cells (17,23). Membrane lipids may affect EL sensitivity, for example, by reducing EL binding to cell membranes or by modifying drug permeability. This latter mechanism was proposed by Ramu *et al.* (24) to explain how tamoxifen and other drugs circumvent multidrug resistance. In fact, they found an increase in PC and a reduction in SPH in multidrug resistant cells after treatment with tamoxifen and other compounds with similar activity.

Of course, there may be other explanations for the different sensitivities of the BL6 variant to EL. For example, other PL, like phosphatidylserine or the fraction containing phosphatidylglycerol and cardiolipid, are also

changed in BL6, in comparison to both F1 and F10 (25), while phosphatidylinositol contents were similar to those of the other high metastatic variant, F10.

It is not clear at present why the contents of certain lipids differ so much in BL6 and the other variants; BL6 growth rate and cell volume were not different from those of F1 and F10 (25). One possibility is that, as BL6 is highly malignant (it retains metastatic capacity after s.c. injection and growth of a primary tumor) it may have developed altered metabolic pathways affecting membrane functions, which may be essential for it to spread and adhere to other tissues.

Endogenous EL have been suggested to play a role in EL cytotoxicity (13,26), but no data are available on their levels in the BL6 variant. A difference in the rate of uptake of EL (by endocytosis) has also been proposed to explain resistance of certain tumor lines (27).

The data confirm that membrane lipids can modulate EL activity. Further studies should give interesting clues as to the relationship between EL cytotoxicity and lipid make up, and may suggest strategies for improving therapeutic efficacy. For example, it may become possible to improve the effectiveness of EL (which at present is very low, at least *in vivo*), by manipulating the lipid composition of target cells. For example, in bone marrow purging, a treatment in which these drugs are currently being tested (28), reducing the CHOL content might increase the sensitivity of leukemic cells to EL. Another possibility, which needs further study with a larger number of tumors, is that by knowing the lipid content of a certain tumor, one could predict whether EL treatment has any likelihood of success. Taking into consideration the observations of Ramu *et al.* (24) and a previous observation by Tsuruo and Fidler (29), who found that among other B16 melanoma variants BL6 was more resistant to conventional antitumor drugs, we suggest that lipid composition-induced modulation of drug sensitivity may be a more general phenomenon that could be exploited to improve antitumor drug efficacy.

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Modulation of Phorbol Ester-Associated Events in Epidermal Cells by Linoleate and Arachidonate

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To elucidate the events elicited by the skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which are modulated by linoleic acid (LA) and arachidonic acid (AA), the activity of these fatty acids in cultured mouse epidermal cells was compared. Approximately 94% of either exogenous radiolabelled fatty acid was incorporated into the total phospholipid pool over 15 h. The relative distribution among the phospholipid classes differed, however, such that approximately 70% of phospholipid-associated [^{14}C]-LA was found in phosphatidylcholine, compared to approximately 30% for [^{14}C]AA. Phosphatidylethanolamine and phosphatidylinositol/phosphatidylserine contained 17 and 13% of the phospholipid [^{14}C]LA, and 34 and 30% of [^{14}C]AA, respectively. Prostaglandin (PG) E_2 production was low but similar in unstimulated cultures prelabelled with either [^{14}C]LA or [^{14}C]AA. However, in cultures treated with TPA (1.6 μM), [^{14}C]AA-prelabelling resulted in approximately three times the amount of [^{14}C]PGE_2 compared with cultures prelabelled with [^{14}C]LA. Cultured cells were found to contain significant Δ6 desaturase activity, which may enable conversion of LA to AA, and thus may account for the observed PGE_2 production from [^{14}C]LA treated cells. AA-Supplemented (1.6 μM) cultures supported approximately twice the induction of ornithine decarboxylase activity by TPA compared with cultures treated with 1.8 μM LA. Activation of partially purified protein kinase C was similar for either fatty acid tested over a 10–300 μM dose range. Overall, the results suggest that LA does not have the same biological activity as AA with regard to several TPA-associated events known to be important in skin tumor promotion. This reduced biological activity of LA may be partly responsible for the known inhibition of mouse skin tumor promotion by high dietary levels of LA [Leyton, J., Lee, M.L., Locniskar, M.F., Belury, M.A., Slaga, T.J., Bechtel, D., and Fischer, S.M. (1991) *Cancer Res. 51*, 907–915].

***Lipids 28*, 407–413 (1993).**

Different types of dietary fatty acids can modulate tumor development in several organ models (1–4). In particular, diets rich in the n-6 fatty acid linoleate (LA; 18:2n-6), have been associated with increased tumor incidence in the pancreas (5), colon (6) and mammary gland (7) of the rat. Additionally, dietary fats containing n-3 fatty acids, such as eicosapentaenoate (20:5n-3) and docosahexaenoate (22:6n-3), have exhibited tumor inhibiting properties in these organs (8–10). In studies of the modulatory effects of dietary lipids on the tumor promotion stage of multistage skin carcinogenesis, we found different effects of n-6 and n-3 fatty acids; increasing the amount of the dietary n-6 fatty acid LA in 15% (w/w) total fat diets correlated with inhibition of tumor promotion (1). In a separate study, a 10% (w/w) fat diet containing n-3 fatty acids in the form of fish oil was shown to not have a significant effect on phorbol ester skin tumor promotion, as compared to an n-6 rich diet (11). With this latter diet LA accumulated in epidermal phospholipids while arachidonic acid (AA; 20:4n-6) decreased slightly, although the ratio of unsaturated to saturated fatty acids did not change appreciably (1). This was somewhat unexpected as LA can be converted to AA in the liver through a series of reactions limited by the first step, Δ6 desaturation. LA and AA can be further metabolized to eicosanoids in the epidermis, including lipoxygenase products of LA and AA (12) and cyclooxygenase products of AA (13).

The metabolites of arachidonate have been shown previously to modulate tumor development in several organs, including the mammary gland (14,15) and skin (reviewed in Ref. 13). Specifically, in multistage skin carcinogenesis in mice, the AA-derived eicosanoid, prostaglandin (PG) E_2 has been found to be required for the induction of ornithine decarboxylase activity (ODC) and epidermal hyperproliferation, events essential to tumor promotion by the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (13,16). We have previously shown that TPA-induced PGE_2 production *in vivo* in the epidermis can be modulated by the level of dietary LA (1). However, unlike mammary tumors where increased dietary LA correlated with increased PGE_2 production (10), we found decreased PGE_2 production in the epidermis of animals fed high levels of LA. The diet-induced decrease in PGE_2 correlated with decreased TPA-induced skin papilloma formation (1).

One concept of multistage carcinogenesis is that it represents a progressive disorder of signal transduction pathways that control gene expression (17). TPA may accomplish this through its action as a highly active analog of the endogenous ligand, diacylglycerol, for protein kinase C (PKC). Fatty acids, including LA and AA, have also been shown to activate specific isozymes of PKC *in vitro* (18–20), and thus may contribute to an altered cell behavior mediated by this signal transduction pathway.

The study reported here was concerned with determining whether the correlation between high dietary LA and decreased skin tumor development correlated with reduced biological activity of LA, as compared to AA, in several TPA-induced responses in epidermal cells. We report here on (i) the rate of incorporation into, and release of these fatty acids from, epidermal membranes; (ii) the amount of PGE produced by cells prelabelled with each fatty acid; (iii) the effect of LA and AA on TPA-induced ODC activity; and (iv) the fatty acid activation of PKC *in vitro*. Understanding the differences in the disposition and activity of LA and AA should contribute to our understanding of the effects of specific dietary fats on skin tumor development.

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Abbreviations: AA, arachidonic acid; ATP, adenosine triphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylureglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; HODE, hydroxyoctadecadienoic acid; LA, linoleic acid; γ-LN, γ-linolenic acid; ODC, ornithine decarboxylase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TLC, thin-layer chromatography; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

MATERIALS AND METHODS

Chemicals. TPA was purchased from Life Systems (Newton, MA) and diluted in acetone. [1-^{14}C]AA, [1-^{14}C]LA (specific activities, 55.7 mCi/mmol and 54.6 mCi/mmol, respectively) and γ-[^{32}P]ATP (23 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Fatty acid methyl esters, adenosine triphosphate (ATP), phenyl methyl sulfonyl fluoride (PMSF), histone, authentic PG and phospholipid standards were purchased from Sigma Chemical Co. (St. Louis, MO). AA, LA and γ-linolenate (γ-LN) standards were obtained from Cayman Chemical Co. (Ann Arbor, MI). Normal phase, Silica Gel 60-254 thin-layer chromatography (TLC) plates (0.25 mm) were obtained from Merck (Darmstadt, Germany); reverse-phase TLC plates were from Analtech (Newark, DE). Solvents and other chemicals were of the highest quality commercially available.

Epidermal cell isolation. Inbred SENCAR (SSIN) mice (21) were obtained from the Science Park Veterinary Division breeding colony (UTMDACC, Smithville, TX). Epidermal cells were isolated from one-day-old newborn mice by the trypsinization procedure of Yuspa and Harris (22). For culture experiments, basal-like cells were plated at densities of 2×10^6 per 35 mm dish, 5×10^6 per 60 mm dish or 15×10^6 per 100 mm dish. 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-111) containing 0.1% fatty acid-free bovine serum albumin but no serum, as described by Morris *et al.* (23). Following plating, the cultures were incubated at 37°C in 5% CO_2 for 24–48 h prior to use in the assays described below.

Phospholipid extraction and analysis. The extent of incorporation of labelled fatty acids was determined by replacing the plating medium with SPRD-111 medium containing radiolabelled LA or AA (0.1 μCi/mL media). At designated times, the medium was aspirated from cultures which were then washed once with cold saline and frozen at −20°C. The thawed cells were scraped into 1.0 mL methanol and a 100-μL aliquot of the cell mixture was counted directly by liquid scintillation. Phospholipids were extracted from the remaining cell mixture with 3.0 mL chloroform/methanol (3:1, vol/vol) and 1.0 mL 2M KCl, and the extracts were dried under nitrogen. Reconstituted extracts as well as authentic standards were separated into phospholipid classes by normal-phase TLC using a solvent system of chloroform/methanol/acetic acid (65:25:4, by vol). Standards were identified with iodine vapors, and corresponding sample phospholipid bands were then scraped and quantitated by liquid scintillation.

Δ6 Desaturase activity. For the analysis of Δ6 desaturase activity in cultured epidermal cells, confluent cultures were grown in serum free SPRD-111 media for 12 to 24 h, after which time cells were scraped and homogenized in ice-cold buffer adjusted to pH 7.0 (24) with 100 mM PMSF, and microsomes were prepared according to the method of Marcel *et al.* (25). Microsomal pellets were resuspended in ice-cold sucrose buffer (0.25 M) with 0.15 M KCl adjusted to pH 7.2. Protein concentrations were determined by the bicinchoninic acid assay (26) system obtained from Pierce Chemicals (Rockford, IL).

Δ6 Desaturase activity was determined in microsomal preparations of cultured cells and adult mouse liver homogenates by measuring the percent conversion of [^{14}C]LA to [^{14}C]γ-LN, according to Chapkin and Ziboh (24). In brief, 2.0 mg microsomal protein was suspended in a potassium phosphate buffer (80 μM, pH 7.2). A duplicate set of microsomal preparations was boiled for 30 min to destroy desaturase activity in order to determine the rate of nonenzymatic conversion of [^{14}C]LA to [^{14}C]γ-LN. [^{14}C]LA was diluted with unlabelled LA to a specific activity of 6.6 mCi/mmole, incubated with the microsomal preparations for 30 min at 37°C and the reaction stopped by the addition of 10.0 mL chloroform/methanol (2:1, vol/vol). Fatty acids were extracted twice, dried under nitrogen and methylated with 6% HCl/methanol (2.0 mL) at 60°C for 2 h. Fatty acid methyl esters were extracted with water/1.0 M aqueous KCl/hexane (1:1:1, by vol) and separated by reverse-phase TLC in a solvent system of acetonitrile/water (90:10, vol/vol) at 40°C. [^{14}C]Fatty acid methyl esters, which co-migrated with authentic standards as identified by autoradiography and staining with iodine vapor, were scraped and quantitated by liquid scintillation counting. The activity of Δ6 desaturase was expressed as percent conversion of [^{14}C]LA to [^{14}C]γ-LN.

TPA-stimulated fatty acid release and PGE synthesis. Release of fatty acids from phospholipids was determined by labelling cultures with either [^{14}C]LA or [^{14}C]AA in SPRD-111 (0.1 μCi/mL medium) for 15 h. The labelled medium was replaced with fresh SPRD-111 containing either acetone or TPA (1.6 μM), and medium was collected at 4 h and quantitated by liquid scintillation counting.

To determine prostaglandin synthesis in viable epidermal cells, cultures were labelled with either fatty acid (0.1 μCi/mL media) for 15 h, washed, then treated with acetone or TPA (1.6 μM) for 4 h. Media were collected and acidified to pH 3 with 1 N HCl and eicosanoids were extracted twice with 3 mL ethyl acetate with extraction efficiencies usually greater than 90%. Extracts were dried under vacuum, redissolved in ethyl acetate and applied with standards to normal-phase silica G TLC plates. Plates were developed with the organic phase of ethyl acetate/isooctane/acetic acid/water (28:13:5:25, by vol) (27). The band which co-migrated with the PGE_2 standard was identified with iodine vapors, scraped and counted. The sample band is referred to as PGE because in the case of [^{14}C]LA labelled cultures this PG may be either PGE_2 or PGE_1.

ODC activity. ODC activity was assessed in cultures pretreated with cold LA or AA (free fatty acid; 1.8 and 1.6 μM, respectively) for 15 h followed by treatment with acetone (1 μL/mL) or TPA (1.6 μM) for 6 h. The concentration of TPA and fatty acid are based on previous studies with this culture system (28). Homogenized scraped cells were centrifuged (12,000 × *g*) in an Na/K-phosphate buffer containing pyridoxal phosphate. ODC activity was determined in the supernatant as described by Weeks and Slaga (29) and total protein was measured by the Coomassie blue reaction (Bio-Rad, Richmond, CA). Results are expressed as nmol CO_2/mg protein/h.

PKC activation in vitro. To determine the relative ability of LA and AA to activate partially purifed epidermal PKC, cells cultured for 24 h were scraped into buffer [pH 7.6; 20 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 2 mM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM dithio-

threitol (DTT), 1 mM PMSF, 0.5% Triton-X, 10 μg/mL pepstatin A, 20 μg/mL aprotinin, and 50 μg/mL leupeptin], homogenized and centrifuged for 10 min in a Beckman TL-100 ultracentrifuge at 541,000 × *g*. After the supernatant, which represents the total cellular extract, was applied to a 1-mL DEAE-cellulose column, the column was washed with 2 column volumes of buffer (without Triton-X 100) and eluted with buffer containing 0.12 M NaCl. The eluate was collected in tubes containing protease inhibitors. The assay used is that described by Ashendel *et al.* (30). Total protein kinase, as well as non-C kinase activity, was determined by comparing the transfer of ^{32}P from γ-[^{32}P]ATP to histone type IIIs in both the presence and the absence of phosphatidylserine (PS) (100 μg/mL) and 2 mM $CaCl_2$. All reaction mixtures contained 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 375 μg/mL histone and 20 μM ATP, along with 0.5–1 × 10^6 dpm γ[^{32}P]ATP. A 75-μL aliquot of the reaction mixture was added to 25 μL of column eluate and allowed to incubate at 30°C for 10 min, after which 50 μL was spotted onto small squares of P81 phosphocellulose paper, immersed in 75 mM phosphoric acid, washed with water, allowed to air dry, and radioactivity was counted. Total counts present in the reaction mixture were determined by counting unwashed paper spotted with 25 μL of reaction mixture. Protein content was also assayed using the Coomassie blue protein assay. From these data, specific activity of PKC was calculated as pmol of phosphate incorporated per min per mg protein. Results are expressed as percent increase in the specific activity of PKC as compared to reactions containing no fatty acid.

Statistical analyses. Data from the above studies were analyzed by the ANOVA Fisher Protected Least Squares Difference test using Statview 512+Software, 1986 (Brain-Power Inc., Calabasas, CA).

RESULTS

Disposition of LA and AA. Because fatty acids are normally found esterified in phospholipids prior to hydrolysis and further metabolism, the equal availability of free LA and AA after agonist activation of cells is dependent on similar patterns of both esterification and hydrolysis. Thus, the extent of incorporation of LA and AA was compared in our serum-free epidermal cell culture system. When confluent cultured epidermal cells were labelled with [^{14}C]LA or [^{14}C]AA, fatty acid uptake was found to increase at the same rate for either fatty acid for up to 15 h ($P > 0.05$) (Fig. 1). Incorporation was nearly maximal by 9 h; for convenience, all further experiments used a 15 h incorporation time.

Comparison of the extent of esterification of the labelled fatty acids into the different lipid classes showed that approximately 94% of either [^{14}C]LA and [^{14}C]AA was incorporated into phospholipids, compared with 6% incorporation into other glycerides for either fatty acid (Fig. 2A). However, the distribution of incorporated LA and AA differed across the phospholipid classes. Of the phospholipid-associated fatty acid, 70% of [^{14}C]LA was incorporated into phosphatidylcholine (PC), and 17 and 13% were in phosphatidylethanolamine (PE) or phosphatidylinositol (PI)/PS (Fig. 2B). [^{14}C]AA was more evenly incorporated into PC, PE or PI/PS (36, 34 and 30%, respectively).

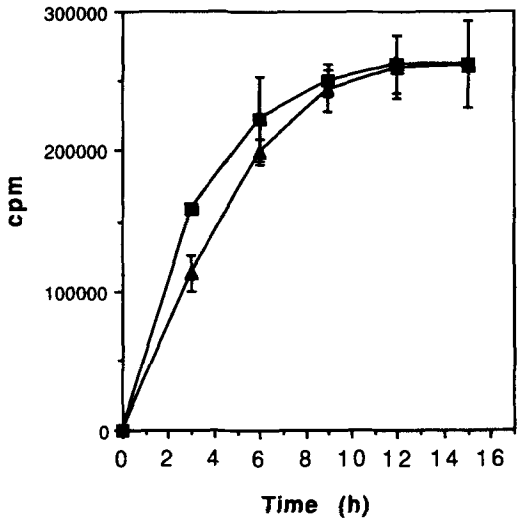

FIG. 1. Rate of incorporation of [^{14}C]arachidonic acid (AA) or [^{14}C]linoleic acid (LA) into total lipids of cultured mouse epidermal cells. Each time point represents cultures (n = 3 or 4) of 2 × 10^6 cells grown for up to 15 h in serum free medium containing 0.1 μCi of [^{14}C]LA (open triangle) or [^{14}C]AA (closed square). Total lipids were extracted and counted. Results are expressed as the mean (n = 3) ± SD of labelled fatty acid incorporated into 2 × 10^6 cells from a representative experiment. Three separate experiments were performed.

As TPA treatment of cultured epidermal cells has been shown to stimulate release of fatty acids from phospholipids *via* phospholipase A_2 activation (31), release of incorporated labelled fatty acids was assessed 4 h after acetone or TPA treatment of cultures (Fig. 3A). Within either the acetone or TPA treatment groups, there was no difference between the amount of radiolabelled LA or AA released. TPA treatment significantly increased ($P < 0.05$) the release of either LA or AA (as fatty acid or metabolites), compared to acetone-treated cultures.

TPA-induced synthesis of radiolabelled PGE was increased significantly ($P < 0.01$) for both fatty acids compared to acetone-treated cultures (Fig. 3B). In AA-prelabelled cultures synthesis of radiolabelled PGE in acetone-treated cultures was significantly higher ($P < 0.01$) at 4 h than synthesis in LA-prelabelled cultures. Within the TPA treatment group, [^{14}C]PGE synthesis was three times greater in [^{14}C]AA-prelabelled cultures than in cultures prelabelled with [^{14}C]LA ($P < 0.01$).

The possibility of LA conversion to AA *via* desaturation-elongation-desaturation was examined by determining Δ6 desaturase activity in cultured epidermal cells. Compared to microsomal preparations from freshmouse liver, cultured epidermal cell microsomes exhibited similar Δ6 desaturase activity measured as the conversion of radiolabelled LA to γ-LN (Table 1). For liver microsomes, an average 10% conversion of LA to γ-LN was observed while microsomes from epidermal cultures supported a 16% conversion.

Biological activity of LA and AA. We have previously shown that AA enhances TPA-induced ODC activity (28); the purpose of this experiment was to determine whether

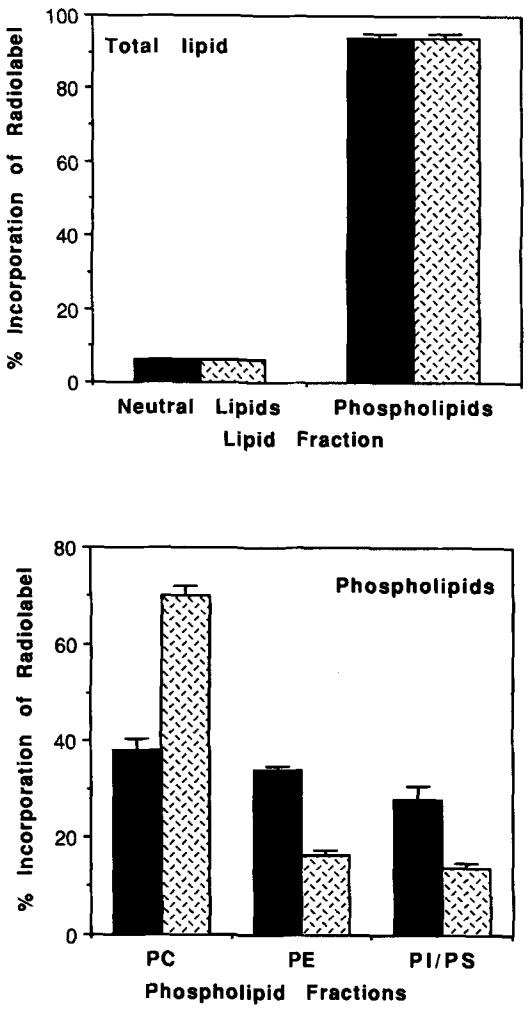

FIG. 2. Incorporation of [^{14}C]arachidonic acid (AA) and [^{14}C]linoleic acid (LA) into epidermal total glycerides and phospholipids. Panel A, following labelling for 15 h with either [^{14}C]AA (solid bars) or [^{14}C]LA (hatched bars) for 15 h, glycerides were separated from phospholipids by thin-layer chromatography (TLC) and counted. Glycerides were tentatively identified as tri-, di- and monoglycerides; sphingomyelin was also identified by co-chromatography with standards. Panel B, phospholipid classes were separated by TLC, identified with authentic standards and counted by liquid scintillation. Values represent the mean cpm ± SD from two separate experiments containing triplicate samples. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

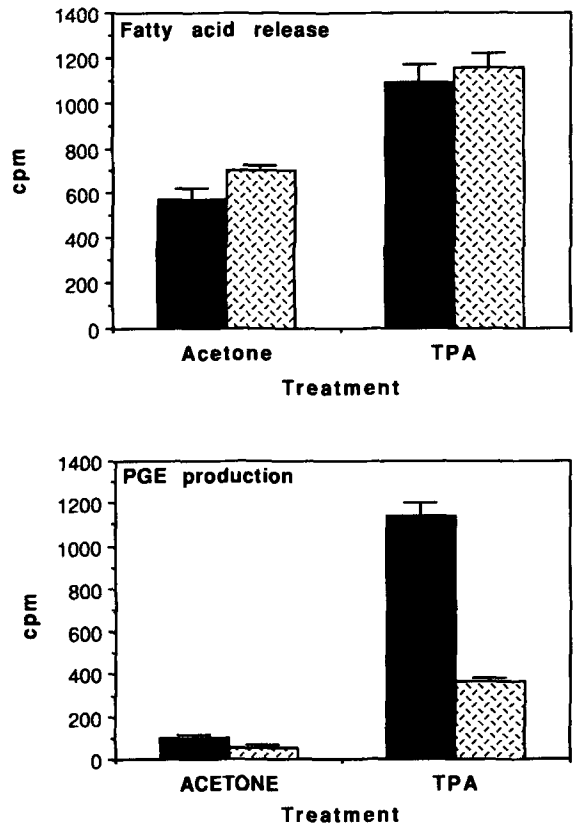

FIG. 3. TPA-Stimulated fatty acid release and PGE production in epidermal cells. After labelling cultures with [^{14}C]AA (solid bars) or [^{14}C]LA (hatched bars) for 15 h, cultures were treated for an additional 4 h with solvent (acetone at 1 μL/mL) or TPA (1.6 μM). Panel A, media was removed and a 100-μL aliquot was counted to determine release of radiolabel. Panel B, PGE production was determined by extraction from 1 mL medium, separation by TLC and counting bands co-migrating with authentic PGE_2 standard. Values represent the mean cpm ± SD from two separate experiments containing triplicate dishes. Abbreviations as in Figure 2; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PG, prostaglandin.

LA supported TPA-induced ODC activity to a greater or a lesser extent than AA. Thus, ODC activity was assessed in cultures supplemented with either cold LA or AA for 15 h followed by treatment with either acetone or TPA for 6 h (Fig. 4). Acetone-treated cultures exhibited very little ODC activity when either fatty acid was present as compared to cultures treated with TPA. Between TPA-treated cultures, AA supported approximately twice the induction of ODC activity compared to cultures grown in LA supplemented media ($P < 0.01$).

Compared to reactions containing no fatty acid, LA and AA activated PKC to a significantly greater extent ($P < 0.01$) (Fig. 5) at concentrations ranging from 10 to 300 μM. Maximal activation of PKC occurred near 100 μM. No difference was observed between the abilities of LA and AA to activate PKC over the dose range tested.

TABLE 1

Δ6 Desaturase Activity in Liver and Cultured Epidermal Cells[a]

Tissue	% Conversion
Liver	10 (9-11)
Epidermal cells	16 (10-22)

[a]Microsomal pellets were prepared from fresh murine liver or epidermal cells grown in serum free media for at least 12 h as described in Materials and Methods. Resuspended microsomal pellets were aerobically incubated at 37°C for 30 min with [^{14}C](linoleic acid). Reactions were stopped by the addition of chloroform/methanol (2:1), fatty acids were extracted, converted to fatty acid methyl esters and separated by reverse-phase thin-layer chromatography. Bands were identified by co-chromatography with authentic fatty acid methyl ester standards, scraped and counted by liquid scintillation. Results are expressed as the average percent conversion (range of three experiments) of [^{14}C]LA to [^{14}C]γ-linolenate.

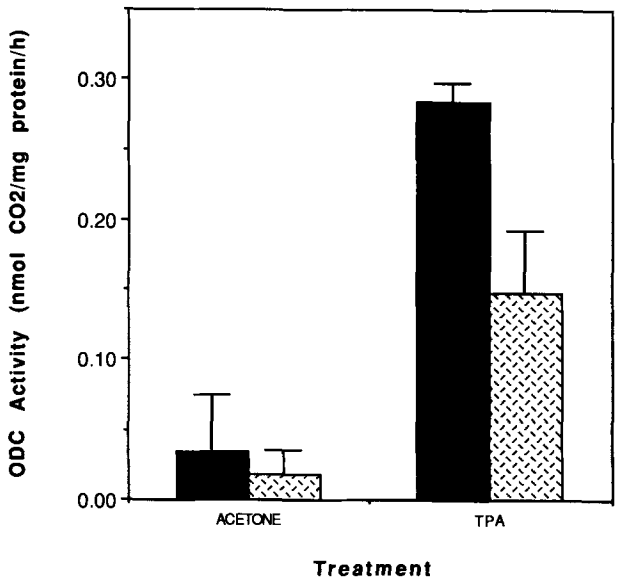

FIG. 4. AA and LA modulation of TPA-induced ornithine decarboxylase (ODC) activity in epidermal cells. Cultures were pretreated with either 1.6 μM AA (solid bars) or 1.8 μM LA (hatched bars) for 15 h followed by treatment with acetone (1 μL/mL) or TPA (1.6 μM) for 6 h. Values represent the mean ± SE for triplicates in two separate experiments. Abbreviations as in Figure 3.

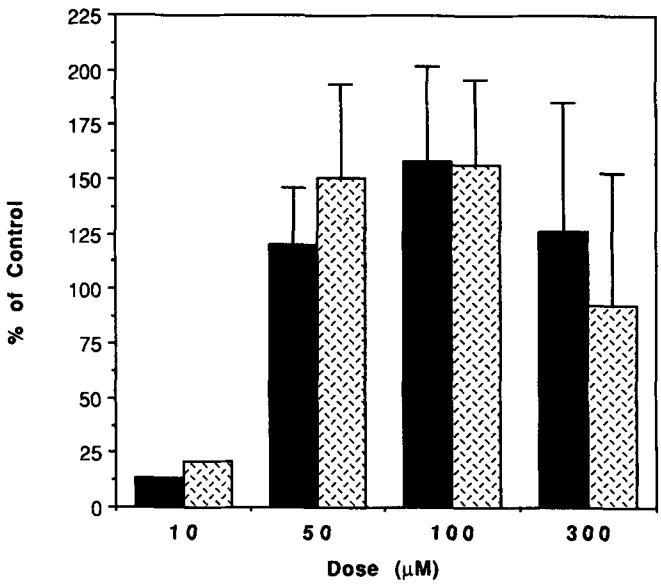

FIG. 5. Protein kinase C (PKC) activation by arachidonic acid or linoleic acid. After partially purifying PKC isolated from cultured epidermal cells, *in vitro* activation by 10, 50, 100, 150 and 300 μM fatty acid was determined. Results are expressed as percent increase in PKC specific activity as compared to assays containing no fatty acid. These data represent the average ± SE of three separate experiments each containing triplicate samples.

DISCUSSION

We have shown that LA, in the form of highly polyunsaturated corn oil, modulates tumor promotion differently in mouse skin (1) compared to mammary tumor development (2). Because fatty acid modulation of mouse tumor development is organ-site specific, we wanted to determine the modulatory roles of the n-6 fatty acids, LA and AA, on specific TPA-elicited events in epidermal cells. In order to answer this question, we traced the rate and lipid class of incorporation and release of radiolabelled fatty acids from cultured epidermal cell phospholipids in cells grown in a serum-free tissue culture system. Where cultured epidermal cells incorporated either exogenously-added fatty acid to the same extent over time, LA was preferentially incorporated into PC while the remainder was incorporated into PE, and PS/PI. Incorporation of AA was more equitably distributed among the four phospholipid classes, the preferential incorporation of labelled LA into epidermal PC is important as this is the major phospholipid which turns over after TPA application to the skin *via* phospholipase A_2 activation (32). Therefore, PC serves as the principal source of free LA available for several metabolic pathways, including lipoxygenase pathways, which have been shown to convert LA to hydroxyoctadecadienoic acids (HODE) in several tissues (33–35), including skin (36). On the other hand, with an elevated pool of free LA, less free AA is available for metabolism to the pro-inflammatory cyclooxygenase and lipoxygenase products known to modulate TPA tumor promotion in mouse skin (13). Hence, LA and its metabolites may have the potential to modulate the TPA-elicited inflammation response which occurs during skin tumor promotion (13).

The rate-limiting step in the conversion of LA to AA, Δ6 desaturase, was found to be active in our cultures to at least the same extent as Δ6 desaturase activity in adult mouse liver. Thus, some of the [^{14}C]LA taken up by the cells in these experiments may have been converted to [^{14}C]AA, although this was not experimentally determined. Conversion of LA to γ-LN by Δ6 desaturation has been previously reported to occur in cultured epidermal cells from neonatal mice and humans (12), but not *in vivo* in guinea pig or mouse epidermis (24,37). This difference between epidermal preparations derived from *in vitro* and *in vivo* sources has important ramifications, especially in the interpretation of these data and in the extrapolation of our findings to *in vivo* fatty acid metabolism and modulation of tumorigenesis in the skin.

The AA-derived prostaglandin, PGE_2, has been shown to modulate several events associated with TPA tumor promotion in mouse skin (13,16,38) including ODC activity, vascular permeability and hyperplasia. We report here that cultures labelled with [^{14}C]LA and treated with TPA supported significantly less radiolabelled PGE production compared with TPA-treated [^{14}C]AA-labelled cells. Because cyclooxygenase, the enzyme involved in the conversion of AA to prostaglandins, has not been shown to use LA as a substrate to form prostaglandins in the skin (36), the small amount of [^{14}C]PGE detected in [^{14}C]LA-supplemented cultures is most likely derived from labelled LA which had been metabolized to either γ-dihomolinolenic acid or AA.

Treatment of the epidermis with TPA has been reported to result in elevated PGE_2 production prior to induction of ODC and epidermal cell hyperproliferation (39,40). Pretreatment of mouse skin with indomethacin inhibited cyclooxygenase activity and thus resulted in inhibition of PGE_2 formation and reduced TPA-induced ODC activity. Induction of ODC was restored if PGE_2 was topically applied to mouse skin with TPA (16). Our studies indicated that *in vitro*, LA supported significantly less

TPA-induced ODC activity compared with AA-supplemented cultures which may be partly due to reduced PGE_2 synthesis. In *in vivo* dietary studies using n-3 rich oils, a reduction in PGE was observed in rat mammary tumors, which correlated with a concurrent reduction in ODC activity (10,41). Reddy and Sugie (42) have also reported a correlation between n-3 rich diets and reduced ODC activity in the colonic mucosa. Similar findings have been reported in the skin (43) where, compared to mice fed corn oil diets, mice fed n-3 fatty acid-rich diets exhibited reduced ultraviolet-induced ODC activity. Although we have previously shown that reduced PGE_2, associated with the synthesis of PGE_3 from eicosapentaenoate, correlated with reduced TPA-induced ODC activity in cultured epidermal cells (29), we did not observe a correlation between dietary n-3 fatty acid reduction of PGE_2 and inhibition of TPA-induced ODC activity in epidermis *in vivo* (11). We are currently investigating the possible modulatory role of high dietary levels of LA on reduced PGE_2 and its relationship to TPA-induction of ODC's activity in mouse skin *in vivo*.

TPA-induced ODC mRNA expression, activity and tumor promotion in mouse epidermis are thought to be mediated by the activation of PKC, the plasma membrane receptor for TPA (29,44). Because fatty acids have been shown to modulate protein kinase activity in several *in vitro* systems (18-20,45), we also investigated the relative abilities of AA or LA to activate PKC isolated from cultured epidermal cells. Both LA and AA were shown to activate PKC equally at all concentrations of fatty acid tested. The similar ability of LA and AA to activate PKC in epidermal cells has been shown in several (45,46), but not all (47) other cell types, probably due to differences in isozyme profiles in different cell types. The ability of LA and AA to equally activate partially purified PKC from cultured epidermal cells suggests that these fatty acids probably do not differentially affect PKC-mediated events by this mechanism in this model system.

In summary, the incorporation of tracer amounts of radiolabelled LA or AA into the phospholipids of cultured epidermal cells was quantitatively similar although LA was preferentially incorporated into PC. With regard to the modulation of two events elicited by phorbol esters, our results indicate that LA is less efficient at supporting PGE production and induction of ODC activity than AA. However, the activation of PKC isolated from cultured epidermal cells was equal for either fatty acid. The reduction of TPA-induced PGE production and ODC activity by LA *in vitro* may at least partially explain the previously observed inhibition by dietary LA of TPA tumor promotion in mouse skin *in vivo* (1).

ACKNOWLEDGMENTS

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Compounds Biologically Similar to Platelet Activating Factor Are Present in Stored Blood Components

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Agents which prime the neutrophil NADPH oxidase develop during routine storage of whole blood and packed red blood cells. This plasma priming activity can be inhibited by bepafant (WEB 2170), a specific platelet activating factor (PAF) receptor antagonist. Quantitation of the priming agent(s), by a commercially available radioimmunoassay for PAF, reproducibly demonstrated high levels of PAF activity. However, analysis of these plasma samples from stored blood components by gas chromatography/mass spectroscopy did not reveal any 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. We conclude that the polyclonal antibody to PAF used in these studies may have recognized different epitopes of a family of heterogeneous, biologically active lipids that manifest their effects through the PAF receptor.

Lipids 28, 415–418 (1993).

Platelet activating factor (PAF) is a biologically active lipid which has been implicated as an etiologic agent in a number of clinical conditions which include necrotizing enterocolitis, asthma and the adult respiratory distress syndrome (ARDS) (1–4). PAF also causes a number of biologic effects on blood cells including both platelet aggregation and polymorphonuclear leukocyte (PMN) priming (5,6). Priming is a process by which an agent augments a response to a subsequent agonist but does not itself activate this response (5,7). PAF not only primes the PMN NADPH oxidase but also augments other PMN functions including adherence, chemotaxis and degranulation (5,7,8). Intracellular synthesis of PAF is upregulated by lipopolysaccharide (LPS) priming of PMN (9). PAF is also thought to be a mediator of inflammation, although its precise role in inflammation is not clear (5,7–9). There are specific PAF receptors on the PMN membrane through which PAF manifests its biologic activity including its own synthesis (6,9). These receptors can be blocked by a number of synthetic molecules in a competitive fashion (10). PAF is rapidly metabolized *in vivo* to form inactive lysoPAF by a plasma acetylhydrolase (11). Recent studies in our laboratory have documented the presence of oxidase priming activity in the plasma from stored blood components (12,13). Developing during storage, this plasma priming capacity of whole blood and packed red blood cells enhanced the NADPH oxidase activity in response to formyl-methionyl-leucyl-phenylalanine (fMLP) 2.2–2.8-fold by the time the components outdated (12,13). Priming was inhibited by bepafant (WEB 2170), a specific PAF receptor antagonist (12,13). In addition, the priming activity was extractable into chloroform which confirmed the lipid nature of this priming substance and suggested that this priming agent was PAF (12,13).

In an effort to quantitate the lipid(s) from stored blood, radioimmunoassays for PAF were performed using a commercially available polyclonal sheep antibody, an antibody which has been previously shown not to react with other acyl or alkyl PAF-like lipids except for C_{16} or C_{18} PAF (14). Gas chromatographic/mass spectrometric (GC/MS) measurements demonstrated that the immunoreactivity was not due to PAF or its 1-*O*-acyl analogue. The results suggested that a number of lipids rapidly prime PMN through the PAF receptor, and also showed PAF immunoreactivity.

MATERIALS AND METHODS

Plasma isolation. Ten healthy volunteers each donated one unit of whole blood: five were left as whole blood (WB) and five were separated into packed red blood cells (PRBC) by standard centrifugation procedures (13). WB and PRBC were stored at 4°C under standard protocols (13). Sterile couplers were inserted into the storage containers, and samples were obtained weekly until outdate of the component, 35 d for WB and 42 d for PRBC. These samples were centrifuged at 7,000 × *g* for 7 min to remove cells followed by a second spin at 12,500 × *g* for 5 min to remove acellular debris. Plasma samples were divided into 1-mL aliquots and stored at −70°C.

Neutrophil priming. PMN were isolated by standard techniques including dextran sedimentation, ficoll-hypaque gradient centrifugation and hypotonic lysis (12). The isolated PMN were pre-incubated for 5 min at 37°C with 400 μM WEB 2170 to block the PAF-receptor. Plasma from the stored components was added so that the final plasma concentration was 10% (by vol). The maximal rate of O_2^- formation was measured in response to fMLP by the superoxide dismutase (SOD) inhibitable reduction of cytochrome c at 550 nm as previously described (12).

Lipid extraction and radioimmunoassay (RIA). The samples were extracted with a chloroform/methanol-0.2% acetic acid/water mixture (1:1:1, by vol), and the chloroform phase was taken to dryness in a rotary evaporator (15). An RIA (New England Nuclear, E.I. Du Pont, Boston, MA) was performed three times on each sample. Briefly, the dried lipids were resolubilized in Tween 20 and incubated with a primary sheep polyclonal antibody to PAF for 15 min at room temperature. The secondary anti-sheep antibody together with ^{125}I-labelled PAF was then added, and the reaction mixture was incubated overnight. The samples were centrifuged at 1000 × *g* for 30 min,

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Abbreviations: ARDS, adult respiratory distress syndrome; [D_3]-PAF, 1-*O*-alkyl-2-[$^2H_3$]acetyl-*sn*-glycero-3-phosphocholine; fMLP, formyl-methioninyl-leucinyl-phenylalanine; [D_3]PAPC, 1-*O*-palmitoyl-2-[$^2H_3$]acetyl-*sn*-glycero-3-phosphocholine; GC/MS, gas chromatography/mass spectrometry; [^{3}H]PAF, 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine; LPS, lipopolysaccharide, endotoxin; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; ODS, octadecylsilica; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAPC, 1-*O*-palmitoyl-2-acetyl-*sn*-glycero-3-phospho-choline; PMN, polymorphonuclear leukocytes; PRBC, packed red blood cells; RIA, radioimmunoassay; SOD, superoxide dismutase; WB, whole blood; WEB 2170, bepafant.

TABLE 1

Priming of the Neutrophil Oxidase by Whole Blood (WB) and Packed Red Blood Cells (PRBC) During Storage

Storage time	O_2^- Production[a]			
	WB	WB + WEB 2170 (400 μM)	PRBC	PRBC + WEB 2170 (400 μM)
Control (Buffer)	2.4 ± 0.2[b]	1.9 ± 0.1	2.7 ± 0.5	2.5 ± 0.4
Day 0	1.9 ± 0.1	1.5 ± 0.2	2.9 ± 0.5	2.8 ± 0.3
Day 7	2.1 ± 0.1	1.5 ± 0.1	2.8 ± 0.8	2.6 ± 0.6
Day 14	2.8 ± 0.6	1.7 ± 0.2	3.2 ± 0.9	2.5 ± 0.7
Day 21	4.1 ± 0.5	2.4 ± 0.3	4.7 ± 1.3	2.8 ± 0.8
Day 28	4.7 ± 0.9	1.8 ± 0.3	6.9 ± 1.3	3.2 ± 1.0
Day 35	5.5 ± 0.6	2.0 ± 0.1	9.0 ± 2.0	3.7 ± 1.2
Day 42	–	–	10.7 ± 1.8	4.5 ± 1.8

[a]Priming activity measured as the maximal rate of O_2^- in response to formyl-methioninyl-leucinyl-phenylalanine as described in Materials and Methods. WEB 2170, bepafant.

[b]Results are nmol/3.75 × 10^5 cells/min and numbers are the means ± the SE of the mean of two units of both WB and PRBC.

the supernatant removed and the pellets counted in a gamma counter. The quantitation of PAF was completed by interpolation from a standard curve of known PAF concentrations quantitated simultaneously by the same RIA procedure. As controls, solutions with either [^{3}H]PAF (1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine; NEN, Du Pont, Boston, MA; specific activity 10Ci/mmol) or known concentrations of PAF were extracted and quantitated by RIA. Quantitation of both sets of controls demonstrated a recovery greater than 90%.

GC/MS. A second aliquot from each specimen was analyzed by GC/MS as previously described in detail (16). Briefly, the isolation procedure involved addition of [D_3]PAF (1-*O*-alkyl-2-[$^2H_3$]-acetyl-*sn*-glycero-3-phosphocholine) and [D_3]PAPC (1-*O*-palmitoyl-2-[$^3H_2$]acetyl-*sn*-glycero-3-phosphocholine) for use as internal standards followed by extraction of the sample with ethanol. The lipid components were then adsorbed on an octadecylsilica (ODS) solid phase extractor cartridge (Bond-Elut, 100 mg, Analytichem, Harbor City, CA). The ODS column was eluted with ethanol, and the extract was put directly on a silica solid phase extractor cartridge (Supelco, Bellefonte, PA). An enriched PAF fraction was eluted, hydrolyzed to diradylglycerol by phospholipase C (Sigma, St. Louis, MO) and derivatized with pentafluorobenzoyl chloride (Aldrich, Milwaukee, WI). The samples were taken to dryness and re-dissolved in 10 μL of decane for injection onto the GC/MS.

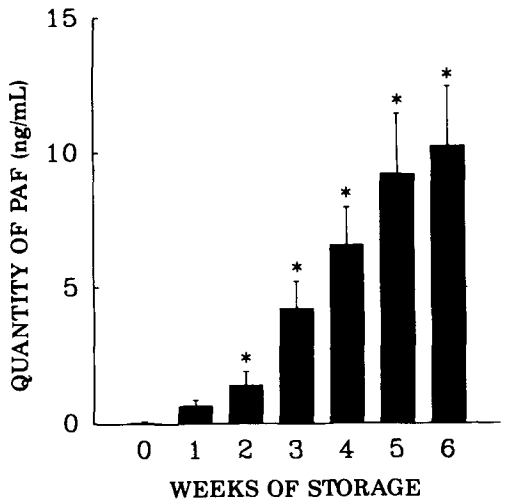

FIG. 1. Quantitation by radioimmunoassay of the amount of platelet activating factor (PAF)-like lipid(s) in plasma samples from stored whole blood. Controls consisting of [^{3}H]PAF and known concentrations of PAF dissolved in fat free albumin prior to lipid extraction demonstrated yields of greater than 90% as described in Materials and Methods. The asterisk denotes statistical significance as compared to the amount of PAF-like lipid quantitated on the day of component collection (day 0, $P < 0.05$).

RESULTS

The PMN priming activity from two units each of both WB and PRBC is shown in Table 1. During routine storage the amount of PMN priming increased dramatically. The maximal plasma priming activity at component outdate was completely inhibited by 40 μM WEB 2170 in samples from WB and by 80 ± 12% in plasma samples from PRBC. Previous results have demonstrated that 400 μM WEB 2170 inhibited purified PAF priming of PMN by 80% in similar experiments (12).

The RIA quantitation of this PAF-like agent that developed over routine storage of all PRBC units is shown in Figure 1. On the day of phlebotomy, the amount of PAF-like lipid estimated by RIA was approximately 50 pg/mL. The amount of this immunoreactive lipid increased with storage to a level equivalent to 10 ng/mL of PAF by the time of product outdate. The amount of lipid became significant following two weeks of storage ($P \leq 0.05$).

Analogous quantitation of the PAF-like lipid was completed for plasma from WB, and the results are shown in Figure 2. As with PRBC, approximately 50 pg/mL of PAF-like lipid was present on the day of isolation. The amount of lipid increased with storage to a maximum amount equivalent to 2 ng/mL of PAF becoming significant by four weeks of storage ($P \leq 0.05$). The PAF immunoreactivity at outdate from PRBC is about 5-fold higher than the corresponding amount of PAF-like lipid from WB.

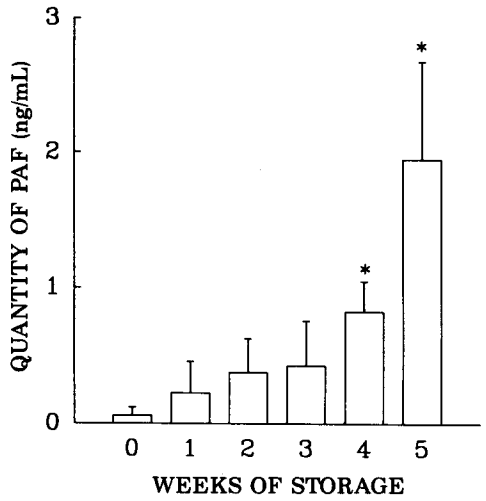

FIG. 2. Quantitation by radioimmunoassay of the amount of platelet activating factor (PAF)-like lipid(s) in plasma samples from stored packed red blood cells. Controls consisting of [^{3}H]PAF and known concentrations of PAF dissolved in fat free albumin prior to lipid extraction demonstrated yields of greater than 90% as described in Materials and Methods. The asterisk denotes statistical significance as compared to the amount of PAF-like lipid quantitated on the day of component collection (day 0, $P < 0.05$).

TABLE 2

Analysis of Plasma Samples from Stored Packed Red Blood Cells (PRBC) and Whole Blood (WB) (at 42 and 35 d, respectively) by Gas Chromatography/Mass Spectroscopy (GC/MS)[a]

Sample	Quantity of lipid	
	PAPC (ng/mL)	PAF (ng/mL)
PRBC1	7.13	<0.1
PRBC2	5.73	<0.1
PRBC3	5.78	<0.1
PRBC4	4.27	<0.1
PRBC5	4.67	<0.1
WB1	9.42	<0.1
WB2	11.90	<0.1
WB3	15.40	<0.1
WB4	14.10	<0.1
WB5	10.40	<0.1

[a]WB and PRBC were collected by phlebotomy, separated and stored by standard techniques. The numbers represent the relative amounts of platelet activating factor (PAF) and PAPC at outdate of the blood products when polymorphonuclear leukocyte priming is maximal. Analysis by GC/MS was completed as described in Materials and Methods. PAPC is 1-*O*-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine.

No hexadecyl or octadecyl PAF was detected by GC/MS in any of the plasma samples from either WB or PRBC on the day of collection or the day of component outdate (35 and 42 d, respectively). The result of GC/MS analysis for the day of outdate of the blood component is presented in Table 2. A PAPC analogue of PAF was present in these plasma samples. Although PAPC has the ability to prime PMN, concentrations 100-fold greater than found in stored PRBC and WB were required to produce PMN effects similar to PAF (16). Moreover, the relative amounts of PAPC on the day of collection of the components were similar to the amounts at component outdate. Since the relative amount of PAPC by GC/MS did not increase over storage while the PMN priming activity did, there was no correspondence between PMN priming activity and PAPC concentrations. Neither PAF nor its 1-*O*-acyl analogue increased with storage of either PRBC or WB (Table 2). Previous results have demonstrated that PAPC has some minor cross-reactivity with the PAF antibody, but the increased immunoreactivity observed in samples from stored blood could not be ascribed to PAPC.

DISCUSSION

PAF is a polar lipid that mediates its actions through a specific receptor in the cell membrane (6,9,17). Several biologic and chemical characteristics of this compound have been utilized to define its presence. Both platelet aggregation and PMN priming by activation of the PAF receptor have been attributed to PAF alone (5,6). This receptor can be competitively blocked by heterazepines, a family of compounds which include WEB 2170 (10). Commercially available polyclonal antibodies to PAF have been reported to be specific for C_{16} or C_{18} PAF (14). PAF behaves chromatographically as a polar phosphatidylcholine and has distinctive mass spectroscopic characteristics (18,19).

PMN priming agents have been implicated in the complications of burns and trauma, including ARDS and multiple organ failure (3,4). These agents prime PMN through the PAF receptor as shown by their inhibition by WEB 2170, a specific PAF receptor antagonist (3,4). Furthermore, blood transfusions, especially massive transfusions (equal to the replacement of the total blood volume), or anaphylactic reactions to blood products, in these patients may be associated with the development of ARDS (19,20). Both PRBC and WB contain a PMN priming activity which can be extracted into lipid solvents and which manifested its activity through the PAF receptor (12–14). It appears likely that this biologically active lipid could play an etiologic role in the clinical complications previously noted.

The data presented here demonstrated production by stored blood of a factor or a class of factors with PAF biologic activity and immunoreactivity. Chemical analysis has shown, however, that the factor is not PAF, which is chemically defined as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. The exact chemical nature of the compound(s) with PAF-like bioactivity is unknown, but the high degree of specificity of both the receptor and antibody binding would imply structural similarities to PAF. The lipids may be generated in stored cellular blood components by metabolic pathways in individual cell types, by cellular interactions or by peroxidation of membrane lipids during blood storage.

It should be noted that many studies have attributed biological activities to PAF on the basis of inhibition of those biologic responses by compounds which are described to be specific PAF receptor antagonists (1,3,4, 8,10,12–14). Such interpretation should be re-examined in the light of this demonstration that compounds which interact with both the neutrophil PAF receptor and a polyclonal antibody to PAF are not necessarily identical to PAF.

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Concentration-Dependent Effects of Eicosapentaenoic Acid on Very Low Density Lipoprotein Secretion by the Isolated Perfused Rat Liver

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The effects of increasing concentrations of eicosapentaenoic acid (20:5n-3; EPA) and oleic acid (18:1n-9; OA) on esterification to triacylglycerols (TG) and phospholipids (PL), and the relationship to formation and secretion of the very low density lipoproteins (VLDL) were compared in the isolated perfused rat liver. Mixtures of EPA and OA were also studied to determine whether substrate levels of one fatty acid might influence the metabolism of the other. The basal perfusion medium, which contained 30% (vol/vol) washed bovine erythrocytes, 6% (wt/vol) bovine serum albumin (BSA), and 100 mg glucose/dL in Krebs-Henseleit bicarbonate buffer (pH 7.4) was recycled through the liver for 2 h. EPA or OA, as a complex with 6% BSA, was infused at rates of 70, 105, 140 and 210 μmol/h. In other experiments, mixtures of EPA and oleic acid (70 μmol total), with molar percentages of 100, 75, 50, 25 and 0% of each fatty acid were infused per hour. BSA (6%) in the buffer was infused alone and served as the control. At an infusion rate of 70 μmol EPA per hour, hepatic VLDL lipid output was not different from that when fatty acid was not infused (approximately half that when 70 μmol OA/h was infused). However, when larger amounts of EPA and OA were infused individually, rates of VLDL secretion were stimulated to a similar extent with either fatty acid. The apparent inhibitory influence of EPA on TG synthesis and VLDL lipid output when 70 μmol EPA were infused per hour could also be overcome by the presence of as little as 25 mol% OA in a mixture. Furthermore, the presence of EPA in the infused fatty acid mixture stimulated the incorporation of OA into TG, enhancing VLDL secretion. When EPA or OA was infused at rates exceeding 70 μmol/h, a constant amount of endogenously-derived fatty acids was incorporated into VLDL-TG, similar in amount to that when exogenous fatty acid was not supplied. However, when EPA was infused at a rate of 70 μmol/h, incorporation of endogenous fatty acids was depressed. At this low rate of EPA infusion, esterification of EPA and endogenous fatty acid was inhibited. Conceivably, this may reflect the existence of independently-regulated pools of fatty acid (exogenous and endogenous), in that only exogenously available fatty acid preferentially enrich the secreted TG. Enrichment of PL by the infused fatty acid at the higher rates of fatty acid infusion showed similar, but much less pronounced, differences between VLDL and liver, compared to that for TG, providing additional evidence for a distinct metabolic pool of PL used for VLDL fabrication. It now appears that when EPA is available to the liver in high enough concentrations, or when OA (or other fatty acids?) is present in substrate amounts along with EPA, competing reactions and/or specific inhibitory influences of EPA on enzymatic reactions are overcome, and EPA can be utilized in a manner similar to OA for esterification to TG with subsequent enhanced VLDL formation and secretion.

Lipids 28, 419–425 (1993).

We reported recently that the utilization of eicosapentaenoic acid (EPA) for synthesis of triacylglycerols (TG) and formation and secretion of very low density lipoprotein (VLDL), by perfused livers from normal male rats was reduced when compared to oleic acid (OA) (1). These animals had been maintained on standard laboratory chow. Generally, similar findings have been reported for studies with rat (2–4) and rabbit (5) hepatocytes in culture, HepG2 cells (6) and isolated perfused rat (7,8) and monkey livers (9,10). Similar data have been obtained with CaCo2 cells (11). Additional studies with animals fed fish oil, a common source of the n-3 fatty acids, suggested that EPA and docosahexaenoic acid (DHA) are poorer substrates for TG synthesis and VLDL formation and secretion than are the more common fatty acids of the n-9 and n-6 series, and may, in fact, be inhibitory to these processes (8). Our observation (1) that DHA was as effective in stimulating TG synthesis and VLDL TG secretion as was OA, however, is, in general, in conflict with the results of other studies carried out in hepatocytes or perfused liver preparations, which indicated (2,9) that both DHA and EPA were poor substrates for esterification to TG secreted in VLDL. Since we had shown that DHA, one of the common n-3 fatty acids, was not inhibitory to VLDL secretion, but that EPA was at a similar concentration, it was of interest to determine if such inhibiting influence was also evident when greater amounts of EPA were available to the liver. In the present study, therefore, we compared the effects of increasing concentration of EPA alone and OA alone, and mixtures of the two fatty acids on TG synthesis and VLDL secretion. It was observed that the apparent inhibition of VLDL secretion, previously reported with EPA, could be overcome when sufficient fatty acid was provided, or when OA was also supplied to the liver for TG synthesis.

MATERIALS AND METHODS

Materials. EPA (>99% purity) was obtained from Cayman Chemical Co. (Ann Arbor, MI); and oleic acid (>99% purity) from Nu-Chek-Prep, Inc. (Elysian, MN). Purity was confirmed by gas chromatography (GC) of the methyl esters (see below). Bovine serum albumin (BSA) (fraction

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Abbreviations: BSA, bovine serum albumin; C, cholesterol; CE, cholesteryl esters; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acids; GC, gas chromatography; OA, oleic acid; PL, phospholipids; TG, triacylglycerols; VLDL, very low density lipoprotein.

V) was purchased from Sigma Chemical Co. (St. Louis, MO). The BSA was delipidated with methanol as described previously (12). All other chemicals were reagent grade.

Liver perfusions. Male Sprague-Dawley rats were obtained from Harlan, Inc. (Indianapolis, IN). Animals (225–250 g) were housed for 1–3 wk with free access to Purina Rodent Chow and water before being used in these studies. Livers from fed animals were perfused *in vitro* as described previously (1) in a recycling system with a medium (80 mL) consisting of washed bovine erythrocytes (30% vol/vol) and purified BSA (3% wt/vol) in Krebs-Henseleit bicarbonate buffer, pH 7.4, gassed continuously with 95/5%-O_2/CO_2. After 20 min equilibration, a pulse of 12, 18, 24 or 36 μmol fatty acid, as a complex with albumin (or albumin in buffer only), was added. During the two-hour perfusion, the complex of fatty acid (OA or EPA) with BSA (6%), or albumin alone, in Mg^{++} and Ca^{++} free buffer was infused at a rate of 11.7 mL/h (1) (70, 105, 140 or 210 μmol fatty acid/hour). These infusion rates permitted us to maintain relatively constant concentrations of free fatty acids (FFA), and yet supply the liver with a threefold range in the amount of OA and EPA. The molar ratios of fatty acid to albumin in the infused complexes ranged from 6.9 to 20.7 with the various rates of fatty acid infusion. However, the actual ratio in the perfusion medium was in the range of 0.8 to 1.5, the result of hepatic uptake of the infused fatty acids. In other experiments, mixtures of 20:5 and 18:1 were infused at 70 μmol/h. A 10-mL sample of the perfusate was removed after 1 h for chemical analysis. At the termination of the experiment, a 60-mL sample was obtained for isolation of the VLDL (13), after sedimentation of the erythrocytes. Lipids were extracted from aliquots of perfusate plasma and VLDL with chloroform/methanol (2:1, vol/vol) using 20 mL/mL plasma (12). At the end of each perfusion experiment, the liver was flushed with 60 mL ice-cold 0.9% NaCl, blotted and weighed. One gram samples were minced and homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in 20 vol of chloroform/methanol (2:1, vol/vol). Individual lipid classes were separated on silica gel thin-layer plates, and assays for TG, phospholipids (PL), cholesterol (C), FFA and cholesteryl esters (CE) were carried out (1).

Analysis of fatty acids by GC was carried out with a Varian 3300 System (Sunnyvale, CA) using an SP2340 column (Supelco, Inc., Bellefonte, PA) (12). Methanolic BF_3 was used to prepare the methyl esters (14).

The rate of hepatic uptake of OA or EPA is expressed as disappearance from the circulating medium and was calculated from the difference between the amount infused and the amount of OA or EPA (GC and mass analyses) remaining at the end of the liver perfusion.

Statistics. All data are presented as means ±SE. The statistical analysis was by a one-way analysis of variance and significance reported for levels of $P < 0.05$. Differences between means were identified using a protected Fisher least significant difference test.

RESULTS

The hepatic uptake of OA and EPA, infused at rates of 70–210 μmol/h for 2 h, is shown in Table 1. No significant differences in uptake between the two fatty acids were observed at any level of infusion. The average concentration of FFA in the perfusate is also presented. Net hepatic secretion of VLDL lipids (Fig. 1) was not statistically different at rates of 70–210 μmol oleic acid/hour during the 2-h perfusion experiment. Although there was a distinct trend indicating an increase in VLDL TG secretion as uptake of oleic acid increased, an apparent maximal output of TG was also evident. We reported previously that with the infusion of OA during 4-h liver perfusions TG secretion positively correlated with the rate of uptake of fatty acid. A maximal output of TG, however, did occur at a rate of OA uptake of about 10 μmol/g liver/h (15). This rate of uptake corresponds to an infusion rate of about 110–120 μmol fatty acid/hour and is approximately the rate at which the maximal output of TG seems to have occurred in the present 2-h liver perfusion studies.

TABLE 1

Uptake by the Perfused Liver and Concentration of OA and EPA in the Medium[a]

Infused fatty acid	Rate of FFA infusion (μmol/h)			
	70	105	140	210
	μmol FFA/mL[b]			
Concentration				
OA (3)	0.47 ± 0.04 (11)	0.55 ± 0.05	0.58 ± 0.07	0.97 ± 0.08
EPA (3)	0.33 ± 0.03 (9)[c]	0.39 ± 0.06	0.53 ± 0.12	0.68 ± 0.07
	μmol/g liver/h			
Hepatic uptake				
OA (3)	5.5 ± 0.6 (11)	8.9 ± 1.8	11.4 ± 1.5	16.4 ± 1.4
EPA (3)	5.9 ± 0.6 (9)	8.9 ± 1.3	12.0 ± 3.0	17.9 ± 2.7

[a]Data are means ±SEM. Numbers of observations are indicated in parentheses. Perfusion conditions are as described in the legend to Figure 1 and the Materials and Methods section.

[b]Average concentration of the infused free fatty acids (FFA) was essentially constant during the two-hour experiment. When exogenous fatty acid was not infused, the average concentration of perfusate total FFA was 0.16 ± 0.02 μmol/mL of which 0.052/μmol/mL was oleic acid (OA) and 0.001 μmol/mL was eicosapentaenoic acid (EPA).

[c]$P < 0.05$ *vs.* OA.

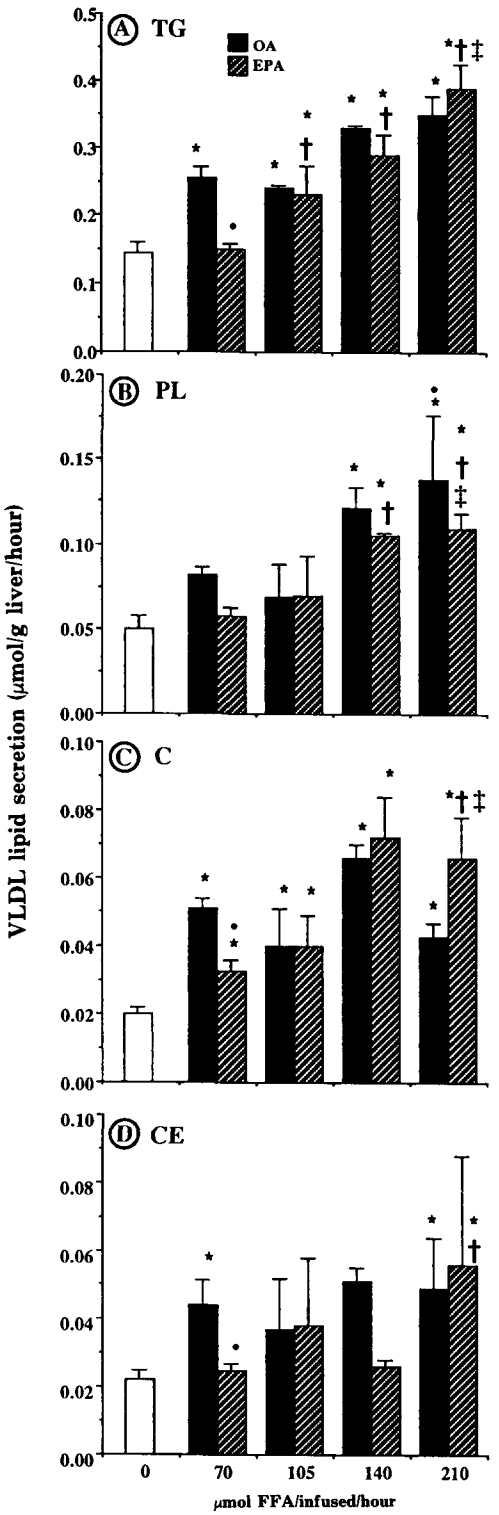

FIG. 1. Effects of eicosapentaenoic acid (EPA) and oleic acid (OA) on secretion of very low density lipoprotein (VLDL) lipids. Liver perfusions were carried out for two hours with a medium containing erythrocytes, as described in the Materials and Methods section. Data are means ±SEM, derived from 3–11 experiments (see Table 1). The symbols indicate: * $P < 0.05$ compared to experiments in which fatty acid was not infused; • $P < 0.05$ compared to infusion of 70 μmol OA/h; † $P < 0.05$ compared to infusion of 70 μmol EPA/h; ‡ $P < 0.05$ compared to infusion of 105 μmol EPA/h. Abbreviations: TG, triacylglycerols; PL, phospholipids; C, cholesterol; CE, cholesteryl esters; FFA, free fatty acids.

With increased infusion of EPA, however, a proportional increase in hepatic output of VLDL lipids was observed (Fig. 1). When EPA was infused at 70 μmol/h, VLDL output was significantly less than that observed with OA and was not different from that when fatty acid was not infused (Fig. 1), as reported previously (1). At higher infusion rates, however, both fatty acids stimulated VLDL output to similar degrees.

Hepatic PL, C and CE concentrations were not significantly affected by the rate of infusion of either fatty acid (24 ± 1, 4.1 ± 0.2 and 0.81 ± 0.05 μmol/g, respectively; $n = 50$). However, after infusion of either fatty acid, hepatic TG concentration was significantly higher (2.73 ± 0.10 μmol/g ($n = 44$) than when exogenous fatty acid was not supplied [1.99 ± 0.15 ($n = 6$)] ($P < 0.05$).

As we reported earlier (12), infused exogenous fatty acid was incorporated preferentially into VLDL-TG and secreted, rather than equilibrating with the large pool of hepatic TG. Table 2 illustrates the enrichment by the infused fatty acid of VLDL and hepatic TG, as infusion rates of OA and EPA were increased. In all cases, incorporation of either EPA or OA into VLDL-TG exceeded that of hepatic TG, indicating the separation of a newly formed metabolic pool and a storage pool, as we have reported for a variety of FFA (12). When the net mass of either infused fatty acid incorporated into the secreted TG was calculated, linearity as a function of infusion rate was evident (Fig. 2). It can also be seen from this figure that the amount of other major fatty acids, linoleate and palmitate (and OA when EPA was infused), present in the TG was similar, regardless of the amount of OA or EPA infused, suggesting a constant synthesis of TG from an endogenous pool, but enriched by additional exogenous fatty acid. The incorporation of infused fatty acid, EPA or OA, into TG, relative to hepatic uptake of fatty acid, is shown in Table 3. It was observed that incorporation of EPA and OA into TG was similar when rates of infusion of FFA were greater than 70 μmol/h; only when infused at a rate of 70 μmol/h was incorporation of EPA less than that of OA in the VLDL, liver or the sum total. Table 4 indicates the similar amounts of endogenously-derived fatty acids (those not infused) which were incorporated into the VLDL-TG with infusion of either exogenous fatty acid; only when EPA was infused at a rate of 70 μmol/h was incorporation of endogenous fatty acid reduced. Infusion of either OA or EPA enriched VLDL and hepatic PL with the infused fatty acid, although much more modestly than it did TG (Table 5).

When various mixtures of OA and EPA were infused (total of 70 μmol/h), secretion of VLDL was stimulated compared to infusion of EPA alone (Fig. 3 and Table 6). With molar ratios for 18:1/20:5 of 75:25 or 50:50, VLDL secretion was stimulated over that when OA was infused alone (Fig. 3). This is evident from incorporation of the fatty acid into the secreted TG. Even with 25% OA, the secretion of TG (Fig. 3) and the incorporation of EPA (Fig. 4) into VLDL-TG (but not liver TG) was enhanced over that when EPA alone was infused. The presence of 25 mol% EPA in the mixture seemed to stimulate the incorporation of OA into TG over that when 70 μmol OA/h (100 mol% OA) was infused (Fig. 4 and Table 6), but statistical significance was not obtained with the smaller number of observations.

TABLE 2

Incorporation of Infused Fatty Acid into VLDL and Hepatic Triacylglycerol[a]

Infused fatty acid	Rate of FFA infusion (μmol/h)				
	0	70	105	140	210
	Total TG fatty acid as OA (%)				
OA					
VLDL	24.3 ± 1.0(5)	46.7 ± 0.8(8)	51.2 ± 4.4(3)	61.9 ± 1.3(3)	63.2 ± 1.1(3)
Liver	26.0 ± 0.4(5)	38.8 ± 0.9(8)[b]	35.0 ± 4.5(3)[b]	49.5 ± 1.0(3)[b]	39.1 ± 3.5(3)[b]
	Total TG fatty acid as EPA (%)				
EPA					
VLDL	3.1 ± 0.1(5)	26.4 ± 1.7(10)	39.2 ± 2.8(3)	46.4 ± 2.0(3)	57.9 ± 0.6(3)
Liver	3.2 ± 0.6(5)	12.3 ± 1.1(10)[b]	12.1 ± 3.3(3)[b]	33.4 ± 6.8(3)[b]	37.9 ± 9.4(3)[b]

[a]Data are means ±SEM and represent percentage of oleic acid (OA) (18:1) or eicosapentaenoic acid (EPA) (20:5). Abbreviations: FFA, free fatty acid; TG, triacylglycerol.
[b]$P < 0.05$ *vs.* corresponding value for very low density lipoprotein (VLDL).

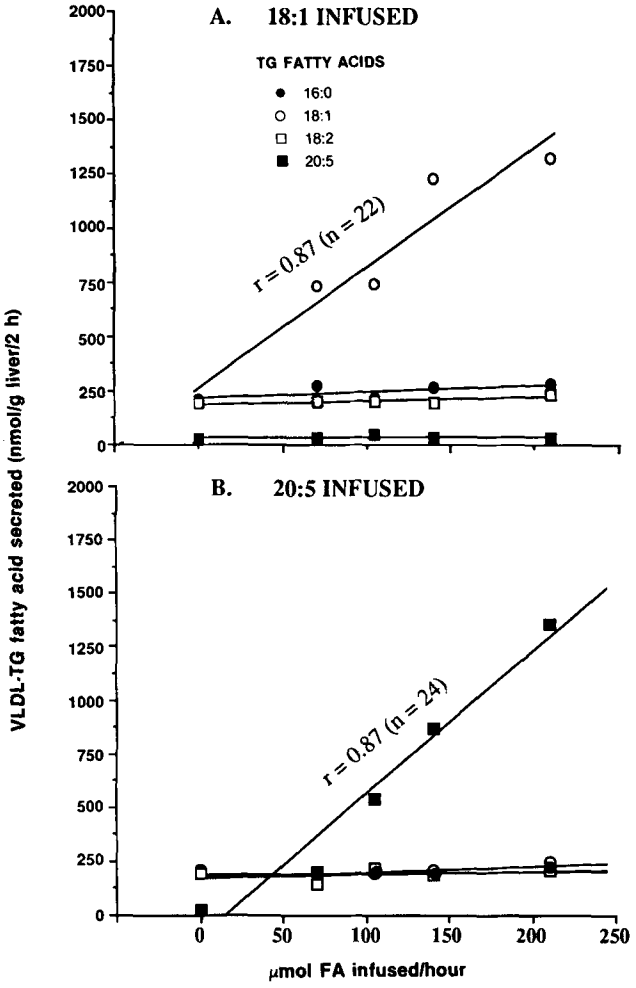

FIG. 2. Effect of infusion rate of oleic acid and eicosapentaenoic acid on secretion of very low density lipoprotein-triacylglycerols (VLDL-TG) fatty acids. The TG fatty acids secreted in the VLDL/g liver/2 hour were calculated as the product of the mean value for TG mass output (Fig. 1) and the percentage of fatty acid in TG for the infused fatty acid (Table 3). Abbreviation: FA, fatty acid.

TABLE 3

Incorporation of Infused Oleic Acid (OA) and EPA into Hepatic and VLDL Triacylglycerol (TG), Relative to Hepatic Uptake of Fatty Acid[a]

Infused fatty acid (n)	VLDL-TG	Liver TG	Total TG
	nmol FFA incorporated/1000 nmol FFA taken up		
OA			
70 μmol/h (8)	71.3 ± 5.1	134.2 ± 31.0	205.4 ± 28.5
>70 μmol/h (9)	54.4 ± 5.8	84.3 ± 14.9	138.6 ± 19.4
EPA			
70 μmol/h (10)	17.9 ± 2.1[b]	50.7 ± 8.4[b]	68.6 ± 10.7[b]
>70 μmol/h (9)	41.3 ± 5.2[c]	76.8 ± 15.3	118.2 ± 19.4[c]

[a]Data are means ±SEM. The values were derived from individual perfusions with the number of experiments given in parentheses. Data points for very low density lipoprotein (VLDL) were calculated from the output of VLDL TG, the percentage composition of the infused fatty acid in the TG (Table 2), and rate at which the fatty acid was taken up (Table 1). For the liver, the calculation was made from hepatic concentration of TG at the end of the 2-h perfusion, the percentage of the infused fatty acid and the rate of which the fatty acid was taken up (Table 1). For liver, the mean (n = 5) amount of each of the infused fatty acids present in TG when fatty acid was not infused was subtracted. Data from experiments in which 105, 140 and 210 μmol fatty acid/h were infused were grouped together since no differences among "groups" were seen (>70 μmol/h). Abbreviations: EPA, eicosapentaenoic acid; FFA, free fatty acids.
[b]$P < 0.05$ *vs.* oleate 70 μmol/h.
[c]$P < 0.05$ *vs.* EPA 70 μmol/h.

DISCUSSION

Our previous report (1) of reduction in utilization of EPA for hepatic TG synthesis, and consequent effects on TG transport in the VLDL, has been reevaluated, using larger amounts of EPA. These current observations indicate a substantial incorporation of EPA into TG similar to that of the reference fatty acid substrate, oleic acid, when EPA becomes available at concentrations greater than examined previously. Generally, our previous data showing re-

TABLE 4

Incorporation of Endogenous Hepatic Fatty Acids into the Secreted VLDL Triacylglycerol (TG) During Infusion of Either OA or EPA[a]

Rate of FFA infusion μmol/h	FA infused	
	OA	EPA
	μmol endogenous FA/g2 h	
None (5)	0.708 ± 0.075	0.904 ± 0.089
70 (8)	0.889 ± 0.053	0.573 ± 0.068(10)[b]
105 (3)	0.771 ± 0.148	0.948 ± 0.293
140 (3)	1.020 ± 0.133	1.388 ± 0.295
210 (3)	0.879 ± 0.070	1.029 ± 0.092

[a]Data are means ±SEM with the number of observations (n) in parentheses. Data were calculated from percentage composition of the total very low density lipoprotein (VLDL) fatty acid (FA) excluding infused oleic acid (OA) or eicosapentaenoic acid (EPA) (Table 2) and the μmol TG FA secreted/2 h (Fig. 1). FFA, free FA.
[b]$P < 0.05$ *vs.* all other groups.

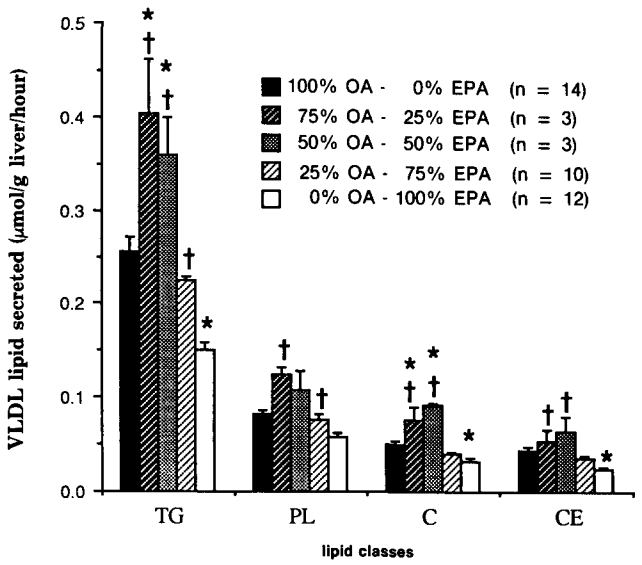

FIG. 3. Effect of oleic acid (OA)/eicosapentaenoic acid (EPA) molar ratio on very low density lipoprotein (VLDL) lipid secretion. Liver perfusions were carried out for two hours with a medium containing erythrocytes, as described in the Materials and Methods section. Seventy μmol of total free fatty acid were infused per hour at the indicated molar percentages. The symbols indicate. *$P < 0.05$ compared to 100% OA; + $P < 0.05$ compared to 100% EPA.

duced secretion of VLDL and TG by EPA are in agreement with those obtained with rat hepatocytes (2–4). Our present data, however, seem to be in conflict with these studies and with those carried out in Hep G2 cells (6,16) and the perfused liver (7) in which EPA was reported to be inhibitory to TG synthesis, expressed in lower rates of esterification to TG, compared to OA. Recently, Lang and Davis (3) reported that synthesis of TG from [^{3}H]glycerol in hepatocytes, in the presence of 1 mM EPA or DHA, was similar to that from OA. The secretion of TG mass, however, was depressed in response to the two n-3 fatty acids as compared to OA. Furthermore, ^{35}S apoB secretion was also impaired. This finding, along with accumulation of hepatic TG, and a similar increase in hepatic apoB content, lead them to conclude that the n-3 fatty acids impair VLDL assembly and secretion, but this did not result from altered rates of synthesis of TG from these fatty acids.

Our data demonstrate that incorporation of either OA or EPA into secreted TG, and accumulation in hepatic TG, can occur at similar rates (Table 3), *i.e.*, total TG synthesis is similar. This observation is therefore in agreement with the data of Lang and Davis (3) which indicated that TG synthesis was the same for OA and EPA except that VLDL secretion in our study was not diminished, as noted in this report. Nevertheless, at the lowest infusion rate we studied, EPA was distinctly inhibitory to TG synthesis and to VLDL secretion, the latter presumably being a consequence of the former.

Previous reports on studies with cultured hepatocytes (5,6) or CaCo2 cells (11) have also emphasized a preferential utilization of EPA over OA in PL formation. Even in perfused liver studies (7) the incorporation of EPA and DHA into hepatic PL has been reported to be quite significant. We did not directly measure PL synthesis; however, the enrichment of hepatic PL content of EPA at the highest rate of infusion was similar to that of OA (Table 5) which would not seem to suggest a preferential utilization pathway for EPA over that of OA. The composition of the pool of secreted PL (VLDL), however, might be indicative of a higher rate of incorporation of EPA, than OA, into newly formed PL since the enrichment noted at the highest rate of fatty acid infusion was indeed greater. The VLDL pool is quite small, however, and more direct measurement needs to be made to establish the signifi-

TABLE 5

Incorporation of Infused Fatty Acid into VLDL and Hepatic Phospholipid[a]

Infused FFA	Rate of FFA infusion (μmol/h)				
	0	70	105	140	210
	Total PL fatty acid as OA (%)				
OA					
VLDL	8.0 ± 0.6 (4)	12.6 ± 0.7 (3)	14.1 (2)	14.9 (2)	11.7 ± 2.8 (3)
Liver	7.7 ± 0.3 (4)	9.2 ± 0.7 (3)	11.5 (2)	11.2 (2)	11.8 (2)
	Total PL fatty acid as EPA (%)				
EPA					
VLDL	2.4 ± 0.4 (4)	2.7 ± 0.4 (3)	8.6 ± 0.6 (3)	5.8 ± 0.9 (3)[b]	17.6 ± 0.7 (3)[b]
Liver	1.3 ± 0.2 (4)	3.2 ± 0.6 (3)	4.8 ± 0.1 (3)[b]	6.2 ± 9.3 (3)	6.5 ± 0.7 (3)[b,c]

[a]Data are means ±SEM. Figures in parentheses indicate the number of observations. Abbreviations: FFA, free fatty acid; OA, oleic acid; PL, phospholipids; EPA, eicosapentaenoic acid.
[b]$P < 0.05$ *vs.* 0 (μmole/h).
[c]$P < 0.05$ *vs.* very low density lipoprotein (VLDL).

TABLE 6

Incorporation of Mixtures of OA and EPA into VLDL and Hepatic TG[a]

Fatty acid	Ratio mol% oleate to mol% EPA infused (70 μmol/h, total)					
	No fatty acid infused (n = 5)	100:0 (n = 8)	75:25 (n = 3)	50:50 (n = 3)	25:75 (n = 10)	0:100 (n = 10)
	Total TG fatty acids (%)					
OA						
VLDL	24.3 ± 1.0	46.7 ± 0.8	43.9 ± 2.4	33.5 ± 3.2	21.5 ± 1.0	17.2 ± 0.6
Liver	38.8 ± 0.9	38.8 ± 0.9	39.5 ± 2.6	33.8 ± 4.2	24.3 ± 1.3	23.9 ± 0.9
EPA						
VLDL	3.1 ± 0.1	1.8 ± 0.2	6.5 ± 0.9	13.1 ± 1.9	22.1 ± 0.8	26.4 ± 1.7
Liver	3.2 ± 0.6	1.4 ± 0.2	3.6 ± 0.5	7.2 ± 1.4	11.0 ± 0.9	12.3 ± 1.1

[a]Data are means ±SEM. Mixtures of oleic acid (OA) and eicosapentaenoic acid (EPA) were infused at a total of 70 μmol/h. For comparison purposes, data from experiments in which fatty acid was not infused, as well as those for 100% EPA or 100% oleate, are included (Table 2). Abbreviations: VLDL, very low density lipoprotein; TG, triacylglycerols.

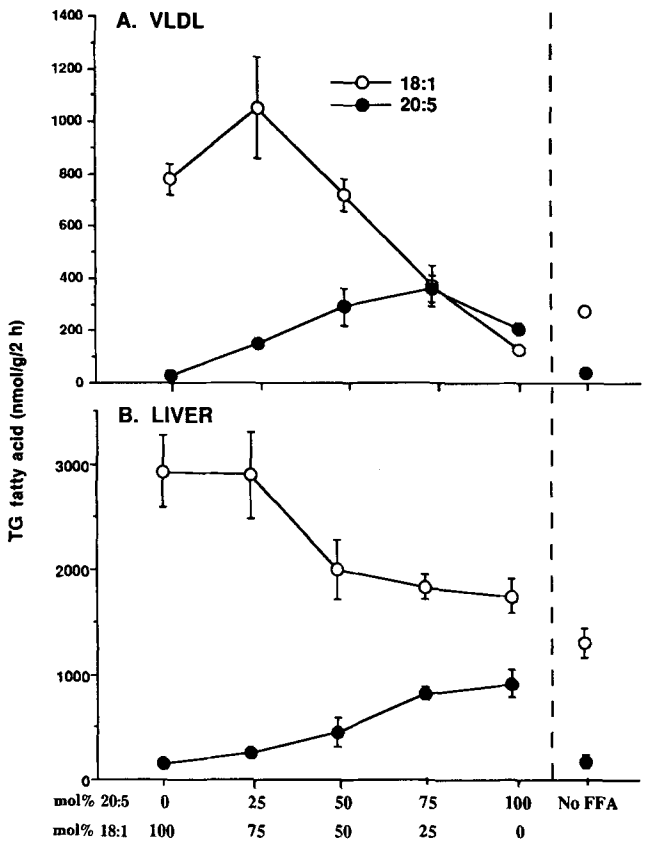

FIG. 4. Effect of oleic acid/eicosapentaenoic acid molar ratio on hepatic and very low density lipoprotein-triacylglycerols (VLDL-TG) fatty acids. Experiments were carried out as indicated in the legend to Figure 3 and the Materials and Methods section. Values were calculated as the product of the individual values for TG mass output (Fig. 3) and the percentage of fatty acid composition of the infused fatty acid in TG. Abbreviation: FFA, free fatty acids.

cance of this observation. Earlier (1) we reported that PL synthesis from [1-^{14}C]EPA and [1-^{14}C]OA was similar, whereas incorporation of OA into TG was greater than was that of EPA, indicating that utilization of EPA for PL synthesis was preferred over that for TG synthesis.

The dilemma surrounding data on the effects of n-3 fatty acids on hepatic VLDL secretion from whole liver and from hepatocytes (HepG2 cells) remains. Consider that the work of Lang and Davis (3), carried out at 1 mM EPA or OA, showed inhibition of VLDL secretion (but not TG synthesis), whereas in the present report, perfusate levels of EPA approaching 1 mM stimulated VLDL secretion, as was observed for OA. Perhaps, the constant supply of the fatty acid as obtained in our perfusion experiments is critical, since in the hepatocyte studies the free fatty acid level would be expected to decrease during incubation. It is also puzzling that we were able to demonstrate that EPA and OA were equally stimulating to TG synthesis and VLDL secretion in the studies with the perfused rat liver model, with amounts of EPA which Wong and Marsh (7) in their liver perfusion studies found inhibitory. Although there were a number of differences in the experimental designs between the two studies, it is not apparent how any of these might be responsible for the difference in results. In general, however, Wong and Marsh (7) found a much greater enrichment of PL and less of TG than we did, which could account for the difference in VLDL synthesis and output. Resolution of the difference must await other unifying evidence not presently available.

In the present liver perfusion studies, as in our previous report (1), there is definite evidence for a persistent secretion of TG derived from endogenous hepatic fatty acids, in agreement with the persistent secretion of endogenous free fatty acids (1). Data in Figure 2 illustrate that a constant amount of two common fatty acids, linoleate and palmitate, are present in secreted TG. As TG secretion increased with infusion of EPA, a constant secretion of OA in TG was also observed. The presence of a constant amount of endogenously-derived fatty acid in the VLDL-TG, regardless of the amount of exogenous fatty acid infused (compared to experiments when FFA was not infused), suggests that the utilization of endogenous and exogenous fatty acid for TG synthesis is independently regulated. This endogenous fatty acid pool presumably is derived from lipolysis (turnover of esterified lipid) and *de novo* synthesis. This consistency in the presence of these two common fatty acids (and small amounts of others) in TG may reflect some restriction in positional

configuration of the fatty acids in the TG, *i.e.*, the liver may be able to produce only TG which contains up to 67% of the individual fatty acid. At the highest rate of OA infusion, a maximum of 63% OA in the TG was obtained, while for EPA the value was similar, 58%, which would seem to support this possibility. Support is also evident in our previous studies with several common long-chain fatty acids in which the greatest enrichment of VLDL-TG was 65% (12).

The mechanisms by which esterification of EPA and secretion of TG in VLDL is enhanced when exogenous OA (at amounts as low as 25 mol% of the 70 μmol of fatty acid mixture infused/hour), or whereby EPA at 25 mol% seemed to enhance OA esterification and TG secretion, are unknown. The observation may reflect the existence of some permissive effect of one fatty acid on another in the process of esterification. Whether this is quite specific, (*e.g.*, for OA and EPA) or is a more general phenomenon is not known, but may relate to limitations on TG structure, as mentioned above.

It is probable that the VLDL particles secreted in response to the infusion of OA or EPA do not differ in size, as indicated by the similarities in ratios (PL + C/TG + CE) of the surface to core lipids (primarily TG) which can be calculated from the data in Figure 1. The overall grouped average of the molar ratios (0.47 ± 0.03; $n = 66$) was not different from that of VLDL produced by the livers of the various individual experimental groups, including that when fatty acid was not provided (0.42 ± 0.06; $n = 6$). This finding suggests that, in these experiments, over the range of TG synthesis and secretion, VLDL fabrication was not curtailed by availability of surface constituents, and that any differences in TG structure (fatty acid composition, up to 63% OA and 59% EPA) do not contribute appreciably to apparent differences in the volume of the secreted particle. The liver will produce and secrete larger VLDL particles, and TG will accumulate in the liver with increasing availability of FFA, perhaps because lipid surface components are not readily available, *i.e.*, PL and/or C (15). We have suggested that C may become limiting (17), resulting in reduced VLDL formation and secretion, and accumulation of TG in the liver. The apparent similarities in particle size as reflected by the lipid ratios in the present study might be predicted, perhaps, because the rate of TG synthesis, reflecting the availability of FFA, seems to be more of a determinant of size (based on these ratios) than is fatty acid structure *per se*, as we suggested previously (12). As we noted previously (12), the molar ratio of the major surface lipids (PL/C) seems to be invariate for the VLDL secreted by the liver. In the present study, the mean value of 66 experiments was 1.98 ± 0.19.

In summary, regardless of the mechanism(s), the inhibition of VLDL secretion observed at low perfusate concentrations of EPA appears to be overcome when increased quantities of EPA are available to the liver, *i.e.*, formation and secretion of VLDL do not differ from that obtained with OA. Furthermore, addition of OA to the medium leads to higher rates of incorporation of EPA into TG. The mechanism(s) which may be involved must be sorted out, whether a reflection of the inhibitory effect on acyl-coenzyme A:1,2-diacylglycerol acyltransferase (18) or phosphatidate phosphohydrolase (6), related to diversion of n-3 fatty acids to PL formation (5,6,11) attended by significant PL compositional differences and related membrane structural modifications; inhibition of lipogenesis (19) or increased oxidation (8), decreased TG transport secondary to defects in VLDL assembly and apoB availability (3,4), or some combination peculiar to species differences or model or experimental design. It is perhaps significant that, only at the lower experimental concentrations of EPA, was VLDL secretion diminished, since it is unlikely that levels of n-3 fatty acid derived from the diet would reach the concentrations seen at the higher infusion rates in our studies.

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Involvement of Vitamin E and Protein Thiols in the Inhibition of Microsomal Lipid Peroxidation by Glutathione

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Iron-ascorbate stimulated lipid peroxidation in rat liver microsomes can be inhibited by glutathione (GSH). The role of protein thiols and vitamin E in this process was studied in liver microsomes isolated from rats fed diets either sufficient or deficient in vitamin E and incubated at 37°C under 100% O_2. Lipid peroxidation was induced by adding 400 μM adenosine 5′-triphosphate, 2.5 to 20 μM $FeCl_3$, and 450 μM ascorbic acid. One mL of the incubation mixture was removed at defined intervals for the measurement of thiobarbituric acid reactive substances (TBARS), protein thiols and vitamin E. In vitamin E sufficient microsomes, the addition of GSH enhanced the lag time prior to the onset of maximal TBARS accumulation and inhibited the loss of vitamin E. Treatment of these microsomes with the protein thiol oxidant diamide resulted in a 56% loss of protein thiols, but did not significantly change vitamin E levels. However, diamide treatment abolished the GSH-mediated protection against TBARS formation and loss of vitamin E during ascorbate-induced peroxidation. Liver microsomes isolated from rats fed a vitamin E deficient diet contained 40-fold less vitamin E and generated levels of TBARS similar to vitamin E sufficient microsomes at a 4-fold lower concentration of iron. GSH did not affect the lag time prior to the onset of maximal TBARS formation in vitamin E deficient microsomes although total TBARS accumulation was inhibited. Similar to what was previously found in vitamin E sufficient microsomes [Palamanda and Kehrer, (1992) *Arch. Biochem. Biophys. 293,* 103-109], GSH prevented the loss of protein thiols in vitamin E deficient microsomes. However, GSH did not protect efficiently against the loss of residual vitamin E in deficient microsomes. These data provide support for the concept that GSH protects against microsomal lipid peroxidation by maintaining protein thiols, and consequently vitamin E, in the reduced state. The lack of protection in vitamin E deficient microsomes may be related to the inability of such low levels of vitamin E to inhibit peroxidation.
***Lipids 28,* 427-431 (1993).**

The oxidation of the polyunsaturated fatty acids associated with membranes can result in the disruption of various cellular functions and cause leakage of intracellular contents. This process, termed lipid peroxidation, is believed to be a major factor in causing various toxicities. The susceptibility of polyunsaturated fatty acids to lipid peroxidation is countered in biological systems by various endogenous antioxidant enzyme systems and antioxidants. Vitamin E is a potent chain breaking antioxidant which inhibits lipid peroxidation in membranes (1). However, since the fully oxidized form of vitamin E is inactive and cannot be used to regenerate the parent molecule, vitamin E turnover is relatively slow, and there is only approximately one molecule of vitamin E per 1200 polyunsaturated fatty acids in membranes (2), it is believed that the partially oxidized tocopherol is reduced to vitamin E in some sort of recycling reaction.

The source of the reducing equivalents which might regenerate vitamin E is unknown. A number of researchers have focused on glutathione (GSH) which is capable of inhibiting lipid peroxidation in rat liver microsomes with the help of a putative heat- and trypsin-labile microsomal "factor" (3-6). This factor appears to exist primarily in rat liver microsomes (7), and there is evidence regarding a soluble factor which may perform a similar or related function (8). The protective effect of GSH against microsomal lipid peroxidation is diminished (9,10) and even abolished (11,12) in microsomes from vitamin E deficient rats indicating a requirement for this vitamin. The microsomal factor also appears to require thiol groups for activity since either *N*-ethyl maleimide or hydroxynonenal treatment of the membrane results in its inactivation (13). However, it is also possible that the protective effects of GSH are related to its direct interaction with protein thiols leading to protection or regeneration of vitamin E.

A time course of the effect of GSH on the loss of vitamin E and protein thiols, and on the formation of thiobarbituric acid reactive substances (TBARS) in vitamin E deficient microsomes undergoing iron-ascorbate stimulated lipid peroxidation, has not been studied in detail. The role of protein thiols is of increasing interest because of the lability of the GSH protective effect to thiol oxidants and recent data indicating the importance of protein thiols in controlling lipid peroxidation (14,15). Furthermore, we have recently shown that heating or trypsinizing microsomes abolishes the GSH-dependent protective effect against protein carbonyl group formation, and this is accompanied by a 60% loss of free protein thiol groups (6).

The present study examined the time course of the effect of GSH on vitamin E oxidation and TBARS formation in hepatic microsomes isolated from vitamin E sufficient and deficient rats. The effect of GSH on vitamin E oxidation during peroxidation in vitamin E sufficient liver microsomes was compared to microsomes pretreated with the thiol oxidant diamide. In addition, the time course of the effect of GSH on the loss of protein thiol groups during peroxidation in vitamin E deficient membranes was studied. The results support the concept that both vitamin E and protein thiols are involved in GSH-mediated protection against lipid peroxidation.

MATERIALS AND METHODS

Materials. Adenosine diphosphate (ADP), butylated hydroxytoluene, *N*-ethylmaleimide, thiobarbituric acid, diamide and 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) were

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Abbreviations: ADP, adenosine 5′ triphosphate; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); GSH, glutathione; TBARS, thiobarbituric acid reactive substances.

obtained from Sigma Chemical Co. (St. Louis, MO). Ferric chloride, trichloroacetic acid, guanidinium HCl and 2,2′-dipyridyl were obtained from Aldrich Chemical Company (Milwaukee, WI). L(+)-ascorbic acid was obtained from MCS Manufacturing Chemists Inc. (Cincinnati, OH). Oxygen was obtained from Big Three Industries Inc. (Laporte, TX). All other chemicals were of reagent grade.

Animals and diets. Two groups of nine rats were maintained for 9 wk on vitamin E sufficient (500 U of vitamin E acetate/g) and vitamin E deficient (0 U of vitamin E acetate/g) AIN-76A diets obtained from Dyets Corporation (Bethlehem, PA). All studies were done in liver microsomes isolated from rats fed these diets. The body weights of rats were monitored during the 9-wk study, and both groups gained weight similarly.

Preparation of microsomes. Microsomal membranes were isolated by differential centrifugation in a 50 mM potassium phosphate buffer (6). Protein was determined by the microbiuret method (16) using bovine serum albumin as the standard. The protein concentration of the final incubation mixture was adjusted to 2.5 mg/mL. Diamide-treated microsomes were prepared by mixing 1 mL of diamide solution (1 mM in 50 mM potassium phosphate, pH 7.4) with 10 mg of microsomal protein in 1 mL. After incubating on ice for 15 min, this mixture was diluted with 15 mL of cold 50 mM potassium phosphate buffer, pH 7.4 and centrifuged at 101,000 × *g* for 60 min. The supernatant was discarded, the pellet gently washed and finally resuspended in 10 mL of the same buffer (1 mg protein/mL). Control microsomes were treated similarly except that diamide was omitted.

Lipid peroxidation assay. Twenty-five mg of microsomal protein was placed in a 25-mL flask in 10 mL of 50 mM potassium phosphate buffer (6). ADP (400 μM) plus 2.5, 10 or 20 μM ferric chloride and 450 μM ascorbic acid (final concentrations) were then added to initiate lipid peroxidation. The mixture was gassed with 100% O_2, stoppered and incubated at 37°C. Lipid peroxidation was quantitated as TBARS, and the lag time prior to the onset of maximal TBARS formation was measured as described before (6).

Protein sulfhydryl (thiol) and vitamin E assays. Protein thiols were determined according to the method of Boyne and Ellman (17). Briefly, 1 mL of the microsomal incubation mixture was precipitated with 0.3 M perchloric acid. After centrifugation, the pellet was washed with 2 mL of 0.3 M perchloric acid, extracted with 8 mL of ethanol/ethyl acetate (1:1, vol/vol) and dried. The lyophilized pellet was dissolved in 6M guanidinium HCl and the absorbance at 412 nm quantitated after adding 1 mL of 2 mM DTNB solution in the presence and absence of 10 μM *N*-ethylmaleimide (6,18).

Vitamin E was extracted from microsomal membranes with heptane, separated on a C_{18} reversed-phase column by high-performance liquid chromatography and measured electrochemically as described previously (19).

Statistics. Results are expressed as the mean ±SE of three separate determinations. Multiple comparisons between groups were done using the Student-Newman-Keuls test (20) after analysis of variance was performed. A paired *t*-test was used to compare mean values at individual time points with and without GSH treatment. A *P* value less than 0.05 was considered significant.

RESULTS

Vitamin E sufficient microsomes subjected to iron (10 μM)-ascorbate stimulated lipid peroxidation showed a rapid time-dependent accumulation in TBARS formation after a 32-min lag period (Fig. 1A). The addition of GSH inhibited TBARS by 84% at the end of the 60 min of incubation in this system. Increasing the iron concentration to 20 μM decreased the lag time before peroxidation in vitamin E sufficient microsomes to 21 min and increased the amount of TBARS formed after 60 min (Fig. 1B). GSH increased the lag time to 26 min in the presence of 20 μM iron and inhibited the maximal rate of TBARS accumulation.

There was a 56% loss of protein thiols in vitamin E sufficient microsomes treated with the thiol oxidant diamide (Table 1). Although diamide treatment had no effect on microsomal vitamin E content (Table 1), the GSH-mediated protection against TBARS formation was abolished (Table 2).

An iron concentration of only 2.5 μM in the presence of vitamin E deficient rat liver microsomes was sufficient

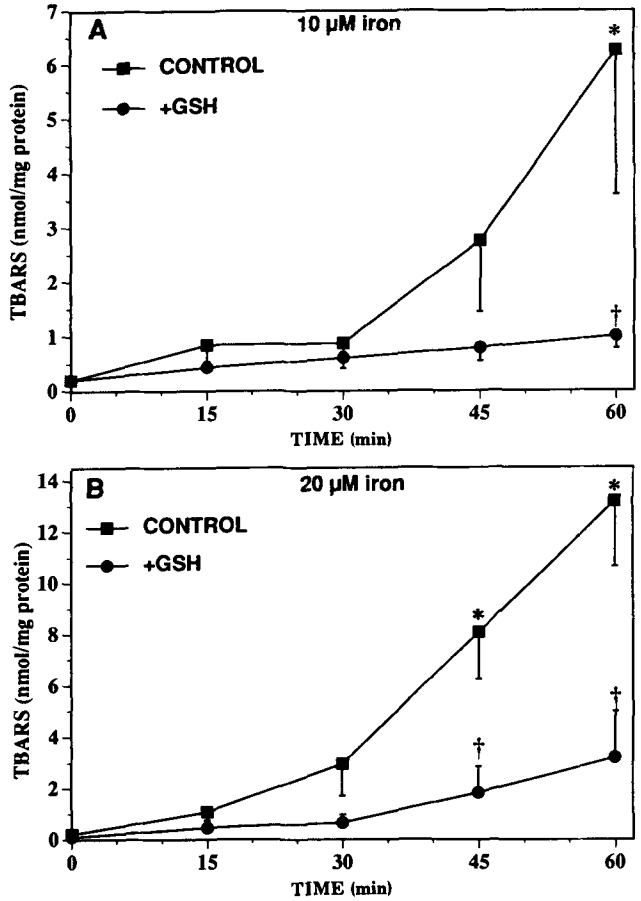

FIG. 1. Time course of thiobarbituric acid reactive substances (TBARS) formation in vitamin E sufficient microsomes incubated with 450 μM ascorbate in the presence and absence of 1 mM glutathione (GSH) and either (A) 10 μM or (B) 20 μM iron. GSH enhanced the lag time before TBARS formation and inhibited total TBARS accumulation at both doses of iron tested. Each result is the mean ±SE of four (A) or three (B) separate determinations. †Significantly different from the coresponding time point lacking GSH. *Significantly different from time 0 ($P < 0.05$).

TABLE 1

Effect of Diamide Pretreatment on Microsomal Vitamin E and Protein Thiol Groups[a]

	Vitamin E (pmol/mg protein)	Protein thiols (nmol GSH equiv./mg protein)
Control	388 (n = 2)	46 ± 4 (n = 3)
Diamide	375 (n = 2)	21 ± 4 (n = 3)[b]

[a]Microsomes (10 mg) were incubated with a 1 mM diamide solution for 15 min at 0°C. Unreacted diamide was removed by washing with 15 mL 50 mM phosphate buffer, pH 7.4, and centrifuging for 60 min at 101,000 × *g*. GSH, glutathione.

[b]Significantly different from control group ($P < 0.05$).

TABLE 2

Effect of Diamide Treatment on GSH-Mediated Protection Against TBARS Formation[a]

Treatment	TBARS (% of control)
Untreated microsomes + GSH	26.3 ± 9.5[b]
Diamide-treated microsomes + GSH	100 ± 9.3

[a]Data are expressed as mean ± SE of three separate determinations. Thiobarbituric acid reactive substances (TBARS) formation was determined in untreated and diamide-treated vitamin E sufficient microsomes incubated at 1 mg protein/mL with 450 μM ascorbate and 10 μM iron for 30 min. TBARS formation in untreated or diamide-treated microsomes in the absence of glutathione (GSH) were not significantly different and represent control values.

[b]Significantly different from respective control ($P < 0.05$).

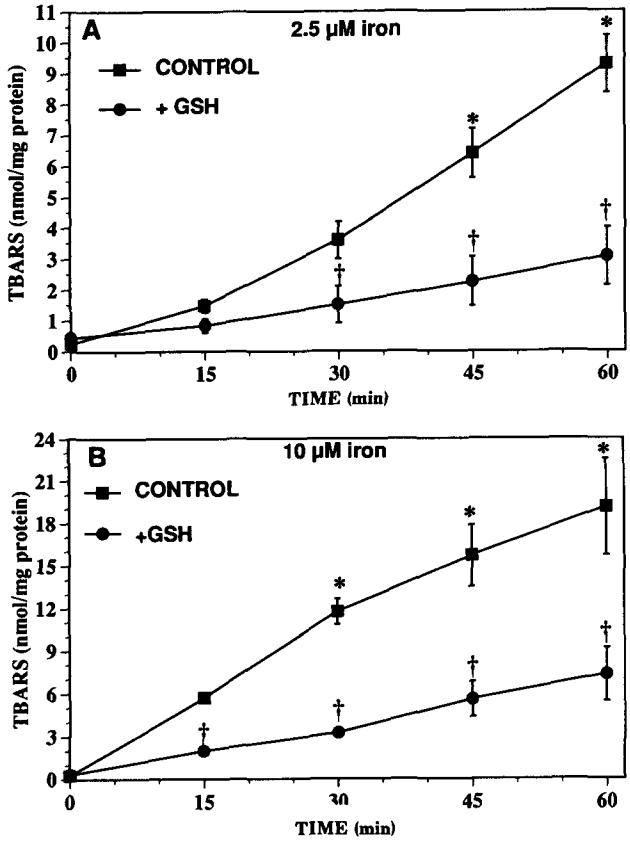

FIG. 2. Time course of thiobarbituric acid reactive substances (TBARS) formation in vitamin E deficient microsomes incubated with 450 μM ascorbate in the presence or absence of 1 mM glutathione (GSH) and either (A) 2.5 μM or (B) 10 μM iron. GSH did not affect the lag time before TBARS formation but inhibited total TBARS accumulation at both doses of iron tested. Each result is the mean ±SE of three separate determinations. †Significantly different from corresponding time point lacking GSH. *Significantly different from time 0 ($P < 0.05$).

to stimulate the formation of the same amount of TBARS (Fig. 2A) as 10 μM iron produced in vitamin E sufficient microsomes (Fig. 1A). In the presence of 10 μM iron, vitamin E deficient microsomes produced about three times as much TBARS (Fig. 2B) as similarly treated vitamin E sufficient microsomes (Fig. 1A). TBARS accumulated in vitamin E deficient microsomes without a distinct lag time prior to the onset of TBARS formation at both doses of iron tested, an effect in contrast to the vitamin E sufficient microsomes. However, GSH significantly inhibited TBARS accumulation in vitamin E deficient microsomes. At the end of 60 min, this inhibition was 67 and 62% compared to control preparations lacking GSH at iron doses of 2.5 and 10 μM, respectively (Fig. 2).

The loss of GSH-mediated inhibition against lipid peroxidation in diamide-treated vitamin E sufficient microsomes was accompanied by the failure of GSH to prevent the loss of vitamin E (Fig. 3A). These results contrast with control microsomes where the addition of GSH resulted in complete protection against the loss of vitamin E throughout the 60 min of incubation.

There was 40-fold less vitamin E present in deficient microsomes as compared to vitamin E sufficient microsomes (Fig. 3B). Although the addition of GSH protected against vitamin E loss at 30 and 45 min of incubation even in vitamin E depleted microsomes, GSH did not completely prevent vitamin E from oxidizing as was the case in the vitamin E sufficient microsomes. In the absence of GSH, approximately 90% of the vitamin E present at time zero was lost by 60 min in both sufficient and deficient microsomes.

Protein thiol groups were gradually lost during peroxidation of vitamin E deficient microsomes at both doses of iron tested (Fig. 4). The addition of GSH prevented the loss of thiol groups throughout the 60 min of incubation with 2.5 μM iron. In contrast, thiol loss was delayed, but not prevented by GSH in the presence of 10 μM iron. Surprisingly, the extent of protein thiol loss was not different at any time point in control microsomes peroxidized with iron-ascorbate and either 2.5 or 10 μM iron.

DISCUSSION

The ability of GSH to inhibit rat liver microsomal lipid peroxidation has been extensively documented (3–7,9–14, 21). GSH appears to require vitamin E to be present in the microsomal membranes since the absence of sufficient vitamin E abolishes GSH-dependent protection against iron-NADPH stimulated lipid peroxidation (11,12). Various reported differences in the effects of GSH on peroxidation may be due to differences in the peroxidation systems employed. It is also possible that other factors

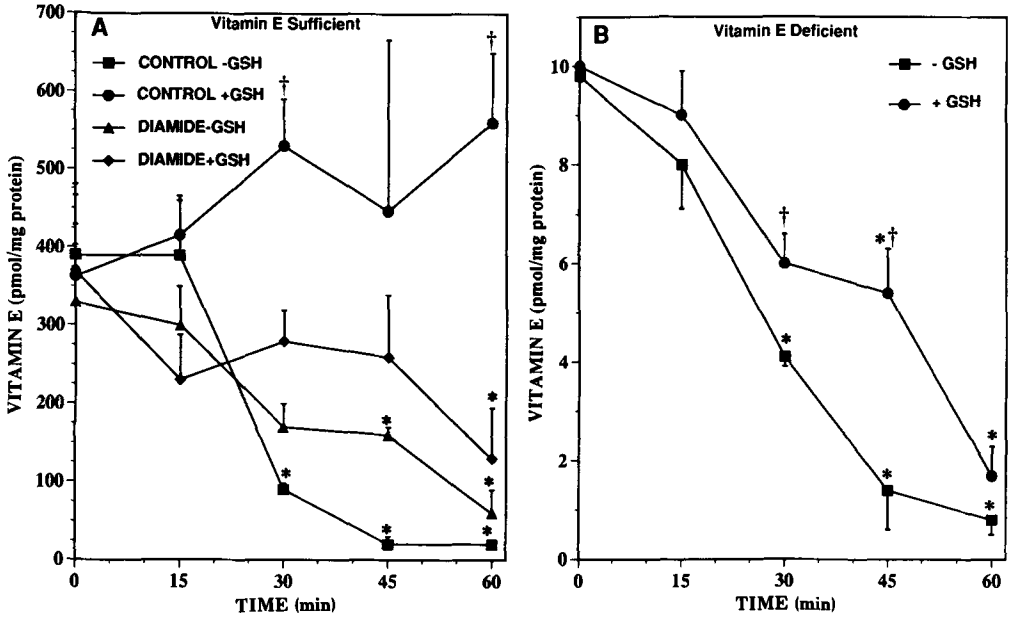

FIG. 3. The time course of the loss of vitamin E in (A) vitamin E sufficient microsomes incubated with 450 μM ascorbate and 10 μM iron in the presence or absence of 1 μM glutathione (GSH) in either control or diamide pretreated microsomes, or (B) vitamin E deficient microsomes incubated with 450 μM ascorbate and 10 μM iron in the presence or absence of 1 mM GSH. GSH inhibited thiobarbituric acid reactive substances formation in control but not in diamide-pretreated microsomes. GSH did not provide complete protection against loss of vitamin E in vitamin E deficient microsomes. Each result is the mean ± SE of three separate determinations. †Significantly different from the corresponding time point lacking GSH. *Significantly different from time 0 ($P < 0.05$).

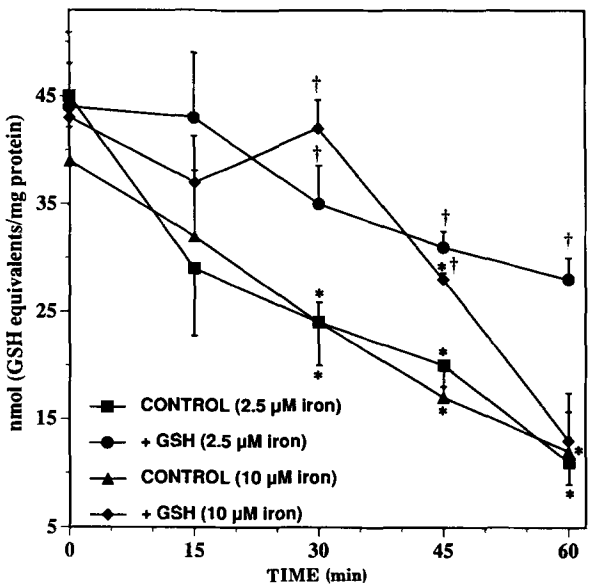

FIG. 4. Time course of loss of protein thiol groups in vitamin E deficient microsomes incubated with 450 μM ascorbate and either 2.5 or 10 μM iron in the presence or absence of 1 mM glutathione (GSH). GSH completely inhibited loss of protein thiol groups at 2.5 μM iron. Each result is the mean ± SE of three separate determinations. †Significantly different from the corresponding time point lacking GSH. *Significantly different from time 0 ($P < 0.05$).

such as the level of E deficiency attained play a role. In addition, while ascorbate exerts prooxidant effect in the presence of a metal (1), it can also scavenge oxygen radicals and regenerate α-tocopherol from the α-tocopheroxy radical (22).

GSH both enhanced the lag time preceding rapid TBARS accumulation and inhibited total TBARS accumulation in vitamin E sufficient rat liver microsomes subjected to iron-ascorbate stimulated peroxidation, in agreement with previous findings (3-7,9-11,13,23). However, when vitamin E sufficient microsomes were pretreated with the protein thiol oxidant diamide, the protective effect of GSH against loss of vitamin E was reduced and against TBARS formation was completely abolished. This indicated that protein thiols play an important role in mediating the GSH-dependent inhibition of lipid peroxidation and in preventing oxidation of vitamin E. These data are consistent with the finding that pretreatment of microsomes with either *N*-ethylmaleimide, 4-hydroxynonenal or glutathione disulfide also compromised GSH-dependent protection against lipid peroxidation (13,24).

Vitamin E deficient microsomes peroxidized without a lag time and at fourfold lower concentrations of iron as compared to vitamin E sufficient microsomes. Furthermore, in contrast to vitamin E sufficient microsomes, the addition of GSH to the vitamin E deficient system did not maintain vitamin E during the incubation period. These data indicate that the level of vitamin E remaining in deficient microsomes was below the threshold required for protection against lipid peroxidation, even for brief periods of time. Alternately, because of accelerated peroxidative damage to these microsomes, the hypothetical microsomal factor could also be destroyed quickly, especially considering its presumed lability to free radicals (21).

Despite the lack of effect of GSH on the lag time prior to the onset of lipid peroxidation, total TBARS accumulation was inhibited by this thiol in vitamin E deficient microsomes. This suggests that GSH was inhibiting lipid peroxidation independent of its effects on vitamin E. Recently, Scholz *et al.* (25) reported that there may be two factors mediating the effect of GSH against lipid peroxidation. One of the factors is vitamin E dependent and the other is vitamin E independent. The exact mechanism by which the second factor interacts with GSH to inhibit peroxidation is unknown. Using an iron-ascorbate system, Hill and Burk (23) demonstrated that GSH could inhibit lipid peroxidation in vitamin E deficient microsomes and have suggested a vitamin E-independent, GSH-dependent radical scavenging activity that could inhibit lipid peroxidation. Reddy *et al.* (9) also observed that GSH inhibited iron-ascorbate stimulated lipid peroxidation in vitamin E deficient microsomes, albeit less efficiently. These findings indicate that the loss of vitamin E can be partially compensated for by the presence of GSH. The mechanism of this effect can be explained by the maintenance of protein thiols in the reduced form by GSH. Our data are consistent with the conclusion that this occurs without the intervention of any undefined factor.

Preservation of reduced protein thiols seems unlikely to account fully for GSH-mediated protection against lipid peroxidation since other thiol agents such as cysteine and mercaptoethanol do not substitute for this protective effect (26). The combination of a protein thiol sparing effect along with the reduction of vitamin E radicals would make GSH a very potent antioxidant molecule against lipid peroxidation in vitamin E sufficient microsomal membranes. The finding that GSH inhibits the peroxidation associated loss of protein thiols, even in vitamin E deficient microsomes, suggests that effects on protein thiols independent of vitamin E content are important in the mechanism by which GSH inhibits microsomal lipid peroxidation.

Takenaka *et al.* (15) have recently examined the relationship between membrane protein thiols and vitamin E. They reported that protein thiols are unable to directly reduce the vitamin E radical to vitamin E. However, protein thiols facing the aqueous cytosol could potentially compete with vitamin E for free radicals formed in an aqueous environment thereby sparing vitamin E from attack and consumption. The same study reported that generation of free radicals in a lipid environment would first cause consumption of vitamin E followed by consumption of protein thiols. In an *in vitro* system, free radicals are generated by the addition of water soluble components such as ascorbic acid and ADP-chelated iron. It is possible that under these conditions protein thiols could effectively compete with vitamin E for free radicals. GSH-dependent protection of protein thiols could thus spare the protein thiols and either prevent vitamin E consumption or perhaps facilitate the transfer of reducing equivalents among GSH, protein thiols and vitamin E.

The data have shown that GSH inhibited lipid peroxidation in liver microsomes from vitamin E sufficient rats more effectively than in liver microsomes from vitamin E deficient rats. The lesser protection in vitamin E deficient microsomes may be related to the inability of residual levels of vitamin E, which were consumed rapidly during peroxidation, to inhibit initiation. In contrast, GSH prevented peroxidation and vitamin E oxidation very effectively in vitamin E sufficient rat liver microsomes. Protein thiols were partially spared by GSH in both vitamin E sufficient (6) and deficient microsomes while destruction of protein thiol groups by diamide abolished GSH-dependent protection against vitamin E oxidation and also lipid peroxidation. The mechanism by which GSH inhibits lipid peroxidation in rat liver microsomes may involve the maintenance of protein thiols, and consequently vitamin E, in the reduced state.

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Mechanism of Decreased Arachidonic Acid in the Renal Cortex of Rats with Diabetes Mellitus

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The purpose of this study was to investigate the roles of decreased synthesis and increased consumption in the depression of arachidonic acid levels in renal cortex and glomeruli of rats with streptozotocin-induced diabetes mellitus. In diabetic rats, arachidonic acid was depressed 33.2% in renal cortex, 47.4% in liver and 66.1% in heart compared to values of control rats. Δ6 Desaturase activity was depressed in renal cortex, liver and heart of diabetic rats to 53.3, 55.5 and 63.7%, respectively, of control values. Δ5 Desaturase activity was also depressed 43.7, 55.5 and 47.6% in renal cortex, liver and heart of diabetic rats, respectively. In other rats the activities of five enzymes involved in the synthesis and esterification of arachidonic acid were measured in renal cortex and in isolated glomeruli. Both tissues from diabetic rats showed depressed activities of Δ5 and Δ6 desaturases, increased activities of long-chain acyl-CoA synthetase and 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase and no change in the activity of elongase as compared to those in control tissues. Malondialdehyde, an end product of lipid peroxidation, was lower in the renal cortex of diabetic rats than in control rats, whereas β-oxidation of linoleic acid and arachidonic acid were similar in diabetic and in control rats. Basal and stimulated prostaglandin E^2 synthesis were significantly higher in isolated glomeruli from diabetic rats compared to those in control rats. In isolated tubules, prostaglandin E_2 synthesis was similarly low in both groups. From these data we conclude that the reduced level of arachidonic acid esterified in lipids of the kidney cortex is caused principally by depressed synthesis of arachidonic acid secondary to decreased activity of Δ5 and Δ6 desaturases. Increased consumption of arachidonic acid to support prostaglandin synthesis may have contributed to the depression of arachidonic acid in glomeruli but not in tubules.
Lipids 28, **433-439 (1993).**

Arachidonic acid, the most abundant constituent polyunsaturated fattty acid in lipids of mammalian cells, has been found to be greatly depressed in a number of organs of humans and animals with diabetes mellitus (1-13). Recent studies in our laboratory have confirmed that arachidonic acid esterified in lipids is depressed in renal cortex of rats with streptozotocin-induced diabetes mellitus (14). Several investigators have shown that the activities of Δ6 and Δ5 desaturases are decreased in the liver of diabetic animals (4,15-18). These observations, together with the demonstration that the rate of conversion of linoleic acid to arachidonic acid is reduced in diabetes mellitus (1,18,19), provide strong support for the hypothesis that the low level of arachidonic acid in complex lipids found in diabetes mellitus is caused, at least in part, by decreased synthesis. In the renal cortex of diabetic rats, however, increasesd activity of Δ6 desaturase activity has been reported (20). This observation implicates a different mechanism underlying the depression of arachidonic acid levels in the kidney.

Arachidonic acid is synthesized from linoleic acid, an essential fatty acid. The five enzymes involved in the synthetic pathway include long-chain acyl-CoA synthetase, which is required for the activation of linoleic and arachidonic acids; Δ6 desaturase, which converts linoleoyl-CoA to γ-linolenoyl-CoA; elongase, which converts γ-linolenoyl-CoA to dihomo-γ-linolenoyl-CoA; Δ5 desaturase, which converts dihomo-γ-linolenoyl-CoA to arachidonoyl-CoA; and acyltransferase, which mediates arachidonoyl-CoA acylation of phospholipids (21). Increased activity of acyl-CoA synthetase has been reported in renal cortex of diabetic rats in one study (20), and increased activity of both acyl-CoA synthetase and acyltransferase has been observed in isolated glomeruli of diabetic rats in another study (22). However, in no single study have the activities of all five enzymes been systematically examined. Thus, no firm conclusions can be drawn about the contributions of decreased arachidonic acid synthesis and/or esterification to the depressed level of arachidonic acid in renal cortical lipids in diabetes mellitus. Furthermore, the possibility that increased consumption contributes to the depression of arachidonic acid in diabetes mellitus has been neglected. The major pathways of arachidonic acid consumption include synthesis of prostaglandins, β-oxidation and free radical mediated lipid peroxidation. The purpose of the current study was to investigate whether the depression of arachidonic acid in the renal cortex of diabetic rats is caused by decreased synthesis or increased consumption of arachidonic acid.

MATERIALS AND METHODS

Diabetes mellitus was induced in male Sprague Dawley rats, weighing 200-250 g, by intravenously injecting streptozotocin at a dose of 65 mg/kg body weight. The rats were studied 4-6 wk thereafter, at which time they manifested hyperglycemia (>500 mg/dL), 4+ glucosuria and polyuria. Three groups of rats were studied. In one group the composition of esterified fatty acids and the activities of Δ6 and Δ5 desaturases were measured in renal cortex, heart and liver of 12 control and of 12 diabetic rats. In a second group the activities of five enzymes involved in the synthesis and esterification of arachidonic acid in lipids and the consumption of arachidonic acid by β-oxidation and lipid peroxidation were measured in the renal cortex of 17 control and 17 diabetic rats. In a third group the above measurements were performed in isolated glomeruli from 6 control and 6 diabetic rats. Rats were anesthetized with 50 mg of pentobarbital, after which

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Abbreviations: DTNB, 5,5-dithio*bis*-2-nitrobenzoic acid; EDTA, ethylenediaminetetraacetate; 1-acyl-GPCAT, 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase; PGE_2, prostaglandin E_2.

they were sacrificed by exsanguination from the aorta. The kidneys were removed and the renal cortex was dissected free. Glomeruli and tubules were isolated by a sieving technique (23). Lipids were extracted from known quantities of tissue by homogenizing first in 5 mL methanol containing 2% HCl. Chloroform was then added to achieve a final concentration of chloroform/methanol/HCl (100:50:1, by vol). All organic solvents contained the antioxidant 2,6-di-*tert*-butyl-4-methyl-phenol at 1.5 mg/100 mL. The homogenate was mixed by vortexing, shaken for 5 min in a water bath at room temperature and filtered over a Whatman glass-fiber filter, model GF/C. The filter was washed with chloroform/methanol (2:1, vol/vol) and the combined filtrates were centrifuged to achieve phase separation. The organic phase was removed and dried under N_2; the residue was re-dissolved in 4 mL of chloroform/methanol (2:1, vol/vol) and stored at −70°C until lipids were analyzed. To determine the fatty acid composition, an aliquot of lipid solution was evaporated to dryness under N_2. Fatty acids were liberated by hydrolysis with formation of methyl esters in the presence of sodium methoxide and methanol. The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography, as previously reported (24).

Enzyme assays. Tissue was homogenized in ice-cold 0.25 M sucrose, 62 mM potassium phosphate, 0.15M KCl, 5 mM $MgCl_2$ and 0.1 mM ethylenediaminetetraacetate (EDTA) at pH 7.0. The homogenate was centrifuged at 16,000 × *g* for 10 min at 4°C, following which the supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The microsomal pellet was resuspended in the homogenizing buffer at a final protein concentration of 10 mg/mL and assayed for enzyme activity. The activities of Δ6 desaturase, Δ5 desaturase and elongase were assayed by adding 0.1 mL of the microsomal suspension (1 mg of protein) to 0.75 mL of buffer composed of 0.25 M sucrose, 0.15 M KCl and 62 mM potassium phosphate (pH 7.4) and containing in micromoles: ATP 10, NADH 26, CoA 1.0, $MgCl_2$ 7.5, glutathione 1.5, NaF 62.5 and NAD 0.5. This assay mixture represents a slight modification of that used by Mandon *et al.* (25). The appropriate [^{14}C]acyl-CoA substrate (50 nmoles) (radiochemical purity 99%, New England Nuclear, Boston, MA) dispersed in 0.15 mL of buffer was added to the above reaction mixture, and the mixture was incubated for 30 min in a shaking water bath maintained at 37°C. The reaction was saponified under N_2 by adding 10% KOH in ethanol and boiling in a water bath for 1 h. After the reaction mixture cooled to room temperature, it was acidified with HCl. The fatty acids were extracted in hexane and then converted to methyl esters with 14% boron trifluoride in methanol (26). The fatty acid methyl esters were separated on 15% $AgNO_3$ thin-layer chromatography plates using hexane/diethyl ether (40:60, vol/vol) as solvent system. The [^{14}C]fatty acid methyl esters were identified by cochromatography with authentic standards and assayed directly on the plate using a radioscanner (Vanguard, Hartford, CT). In pilot experiments, we established that the recovery of fatty acids as methyl esters using this procedure was 94.0 ± 0.7% (n = 4). The data were not corrected to a recovery of 100%. In other pilot experiments we established that under these assay conditions product formation by each enzyme was linear during the 30-min incubation period using samples containing up to 1.5 mg of protein.

Acyl-CoA synthetase activity in the homogenate of renal cortex and of glomeruli was determined as reported by Morisaki *et al.* (27). The homogenate was centrifuged at 800 × *g* for 5 min, and the supernatant was used for enzyme assay. An aliquot of homogenate supernatant containing 10 μg of protein was added to the reaction mixture containing 0.15 M Tris HCl (pH 7.4), 0.1% Triton X-100, 50 mM $MgCl_2$, 20 mM ATP, 0.2 mM [1-^{14}C]fatty acid (radiochemical purity 99%, New England Nuclear), 0.2 mM CoASH and 2.5 mM glutathione in a final volume of 0.25 mL; the mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 2.5 mL of isopropanol/heptane/1N H_2SO_4 (40:10:1, by vol) and the precipitate was removed by centrifugation. Water and heptane were added to the supernatant. The aqueous phase was removed and assayed for [1-^{14}C]acyl-CoA in a liquid scintillation spectrometer. In pilot studies we established that under these assay conditions product formation was linear over 10 min.

1-Acyl-*sn*-glycero-3-phosphocholine acyltransferase (GPCAT) activity was measured in the microsomal fraction of renal cortex and of isolated glomeruli using a spectrophotometric assay as described by Dang *et al.* (28). The method involves the reduction of 5,5-dithio*bis*-2-nitrobenzoic acid (DTNB) by the thio group of free CoASH released from acyl-CoA. An aliquot of the microsomal fraction containing 0.1 mg of protein was added to the reaction mixture composed of 0.1 M Tris HCl (pH 7.4), 0.33 mM DTNB, 0.1 mM 1-acyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids, Birmingham, AL) and 25 μM acyl-CoA. In pilot experiments we established that under these assay conditions product formation was linear for 15 min when samples containing up to 0.15 mg of protein were added.

β-Oxidation was determined by measuring acyloxidase activity in renal cortex homogenized in buffer composed of 0.25 M sucrose, 62 mM potassium phosphate, 0.15 M KCl, 5 mM $MgCl_2$, 0.1 M EDTA and 3 mM imidazole (29). An aliquot (100 μL) of homogenate was added to 1 mL of a reaction mixture composed of 0.25 M sucrose, 0.1 M potassium phosphate, 0.15 M KCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM NADH, 0.5 mM NAD, 1.5 mM glutathione and 45 mM NaF at pH 7.4 and 37°C. The reaction was initiated by the addition of 100 nmol [1-^{14}C]acyl-CoA dispersed in homogenizing buffer and was terminated after 10 min by the addition of acid. The reaction product, $^{14}CO_2$, was trapped in hyamine hydroxide contained in a center well and subsequently assayed in a liquid scintillation spectrometer.

Lipid peroxidation in renal cortex and in glomeruli was estimated by measuring malondialdehyde by the thiobarbituric acid method as previously reported (30).

For determination of prostaglandin E_2 (PGE_2), isolated glomeruli and isolated renal cortical tubules were homogenized in 50 mM phosphate buffer containing 0.01 M EDTA, 0.05% Triton X-100, 0.05% sodium azide and 0.1 mM $CaCl_2$. Aliquots of homogenate were incubated in a water bath at 37°C for 10 min and then assayed for basal PGE_2 synthesis by radioimmunoassay (23). A23187 at 10^{-6}M was added to parallel samples and 10 min later stimulated PGE_2 synthesis was measured.

The data in the text, tables and figures are expressed as the mean ± SE. Differences between groups were assessed by Student's *t*-test. A P value ⩽ 0.05 was considered statistically significant.

RESULTS

The composition of fatty acids esterified in total lipids of renal cortex, liver and heart is summarized in Table 1. Arachidonic acid comprised 28.0 ± 1.1, 21.2 ± 1.0 and 27.0 ± 1.1 mol% of the fatty acids extracted from the renal cortex, liver and heart, respectively, of nondiabetic control rats. In diabetic rats arachidonic acid was significantly reduced by 33.2% in renal cortex, by 47.2% in liver and by 66.7% in heart as compared to the respective control values ($P < 0.01$). The depression of arachidonic acid was accompanied by significant increases of fatty acids that serve as precursors for the synthesis of arachidonic acid. These included dihomo-γ-linolenic acid and γ-linolenic acid in renal cortex and heart, and linoleic acid in renal cortex, liver and heart (Table 1). Isolated glomeruli and isolated renal cortical tubules of diabetic rats showed changes in fatty acid composition similar to those observed for the whole renal cortex (data not shown).

The activities of Δ6 and Δ5 desaturases in renal cortex, liver and heart of control and diabetic rats are summarized in Table 2. In control rats Δ6 desaturase activity in the liver was 5.5-fold higher than in renal cortex and 6.5-fold higher than in heart. In diabetic rats, Δ6 desaturase activity was significantly reduced in all three tissues ($P < 0.01$): −46.7% in renal cortex; −44.5% in liver; −36.3% in heart (Table 2). Δ5 Desaturase activity in the liver of control rats was fourfold higher than in renal cortex and fivefold higher than in heart. In diabetic rats the activity of Δ5 desaturase activity was significantly reduced by 56.2% in renal cortex, by 44.5% in liver and by 52.4% in heart as compared to the respective control values ($P < 0.01$).

In a second group of rats the activities of five enzymes involved in the synthesis and esterification of arachidonic acid in lipids, as well as the consumption of arachidonic acid by β-oxidation and by lipid peroxidation, were measured in renal cortex. The activity of long-chain acyl-CoA synthetase was significantly higher in the renal cortex of diabetic rats than in that of control rats (Table 3). With linoleic acid as substrate, acyl-CoA synthetase activity was 158.4% of that of control ($P < 0.01$); with arachidonic acid as substrate, acyl-CoA synthetase activity was 139.2% of that of controls ($P < 0.01$). The activities of Δ6 and Δ5 desaturases in diabetic rats were depressed 80.2 and 66.7%, respectively, below the desaturase activities of control rats (Table 3). The activity of elongase, which catalyzes the elongation of γ-linolenic acid to dihomo-γ-linolenic acid, was slightly higher in diabetic rats compared to that in control rats, but the difference did not reach statistical significance ($0.05 < P < 0.1$). The activity of 1-acyl-GPCAT in diabetic rats was increased to 161.6% of that of control rats with linoleoyl-CoA as substrate ($P < 0.01$) and to 149.7% of that of control rats with

TABLE 1

Fatty Acid Composition of Lipids in Renal Cortex, Liver and Heart from Control and Diabetic Rats[a]

Fatty acid	Renal cortex		Liver		Heart	
	C	D	C	D	C	D
Tetradecanoic (14:0)	0.29 ± 0.03	0.38 ± 0.04	0.40 ± 0.06	0.55 ± 0.11	0.31 ± 0.05	0.21 ± 0.03
Hexadecanoic (16:0)	17.0 ± 0.2	18.7[b] ± 0.5	20.2 ± 0.6	23.8[c] ± 0.7	14.6 ± 0.5	13.9 ± 0.2
Hexadecenoic (16:1)	0.92 ± 0.11	0.58[b] ± 0.06	2.1 ± 0.1	2.0 ± 0.4	1.3 ± 0.4	0.69[c] ± 0.07
Octadecanoic (18:0)	20.4 ± 0.3	18.6[c] ± 0.4	16.4 ± 0.6	19.4[c] ± 0.7	16.9 ± 0.6	19.0[b] ± 0.5
Octadecenoic (18:1n-9)	9.9 ± 0.3	11.5[c] ± 0.2	13.1 ± 0.4	15.7[b] ± 0.3	12.3 ± 0.5	15.7[c] ± 0.5
Octadecadienoic (18:2n-6)	16.9 ± 0.7	22.3[c] ± 0.6	16.1 ± 0.4	19.6[c] ± 0.8	16.5 ± 1.0	29.2[c] ± 0.7
Octadecatrienoic (18:3n-6)	0.19 ± 0.03	0.46[c] ± 0.04	0.65 ± 0.12	0.44 ± 0.04	0.52 ± 0.06	0.69[b] ± 0.03
Eicosanoic (20:0)	0.26 ± 0.04	0.36 ± 0.06	1.0 ± 0.2	0.47[c] ± 0.06	2.9 ± 0.5	1.7 ± 0.4
Eicosatrienoic (20:3n-6)	1.1 ± 0.1	1.8[b] ± 0.2	2.5 ± 0.4	2.5 ± 0.6	0.04 ± 0.04	4.5[c] ± 0.6
Eicosatetraenoic (20:4n-6)	28.0 ± 1.1	18.7[c] ± 0.5	21.2 ± 1.0	11.2[c] ± 0.7	27.0 ± 1.1	9.0[c] ± 0.3
Docosatetraenoic (22:4)	0.92 ± 0.34	1.7 ± 0.4	0.8 ± 0.1	1.0 ± 0.1	0.02 ± 0.01	0.31[c] ± 0.08
Docosapentaenoic (22:5)	1.1 ± 0.3	1.8 ± 0.3	1.4 ± 0.2	0.79[c] ± 0.07	1.0 ± 0.3	0.82 ± 0.15
Docosahexaenoic (22:6)	0.02 ± 0.01	0.04 ± 0.02	0.53 ± 0.12	0.34 ± 0.03	0.80 ± 0.15	0.14 ± 0.07

[a]Data represent mean ± SE, n = 12, expressed in units of mole percent; C, control; D, diabetic.
[b,c]Significantly different from control, $P < 0.05$ and $P < 0.01$, respectively.

TABLE 2

Desaturase Activities in Renal Cortex, Liver and Heart from Control and Diabetic Rats[a]

	Renal cortex		Liver		Heart	
	C	D	C	D	C	D
Δ6 Desaturase (nmol/mg protein/30 min)	1.07 ± 0.03	0.57[b] ± 0.02	5.93 ± 0.19	3.29[b] ± 0.14	0.91 ± 0.03	0.58[b] ± 0.01
Δ5 Desaturase (nmol/mg protein/30 min)	0.80 ± 0.04	0.35[b] ± 0.02	3.30 ± 0.27	1.83[b] ± 0.08	0.63 ± 0.06	0.30[b] ± 0.04

[a]Data represent mean ± SE, n = 12; C, control; D, diabetic. The microsomal fraction was used to assay desaturases.
[b]Significantly different from control, $P < 0.01$.

TABLE 3

Enzyme Activities in Renal Cortex and Glomeruli of Control and Diabetic Rats[a]

	Renal cortex		Glomeruli	
	C	D	C	D
Acyl-CoA synthetase (18:2)	67.1 ± 2.2 (12)	106.3 ± 4.3[b]	54.5 ± 3.5 (6)	84.2 ± 2.6[b]
Acyl-CoA synthetase (20:4)	60.2 ± 2.0 (12)	83.8 ± 2.7[b]	43.8 ± 2.8 (6)	85.4 ± 2.7[b]
Δ6 Desaturase	0.78 ± 0.08 (17)	0.15 ± 0.01[b]	0.71 ± 0.07 (6)	0.27 ± 0.09[b]
Elongase	0.81 ± 0.11 (17)	1.16 ± 0.15	0.56 ± 0.05 (6)	0.56 ± 0.07
Δ5 Desaturase	0.72 ± 0.09 (17)	0.24 ± 0.04[b]	0.49 ± 0.06 (6)	0.17 ± 0.06[b]
1-Acyl-GPCAT (18:2)	36.7 ± 2.7 (12)	59.3 ± 2.5[b]	31.2 ± 3.0 (6)	47.3 ± 2.5[b]
1-Acyl-GPCAT (20:4)	73.8 ± 2.0 (12)	110.5 ± 3.5[b]	53.4 ± 2.5 (6)	72.8 ± 3.2[b]
Acyl-CoA oxidase (18:2)	0.23 ± 0.01 (17)	0.24 ± 0.01	0.17 ± 0.03 (6)	0.17 ± 0.05
Acyl-CoA oxidase (20:4)	0.23 ± 0.01 (17)	0.21 ± 0.01	0.20 ± 0.05 (6)	0.18 ± 0.05

[a]Data represent mean ± SE in units of nmol/mg protein, except for acyl-CoA oxidase for which the unit is pmol CO_2/mg protein. Assays for desaturases, elongase and 1-acyl-GPCAT were performed using the microsomal fraction, and assays for acyl-CoA synthetase and acyl-CoA oxidase using homogenate of renal cortex and glomeruli; C, control; D, diabetic. Numbers in parentheses denote n. GPCAT, *sn*-glycero-3-phosphocholine acyltransferase.

[b]Significantly different from control, $P < 0.01$.

arachidonyl-CoA as substrate ($P < 0.01$). Thus, of the five enzymes examined, only Δ6 and δ5 desaturase activities were found to be depressed in the renal cortex of diabetic rats. Similar directional changes in the activities of these five enzymes were found in isolated glomeruli of diabetic rats (Table 3).

β-Oxidation of fatty acids by acyl-CoA oxidase is illustrated in Figure 1. Acyl-CoA oxidase activity in the renal cortex of diabetic rats was significantly higher than that of control rats when palmitoyl-CoA or oleoyl-CoA were the substrates; however, acyl-CoA oxidase activity in diabetic rats was not different from control when linoleoyl-CoA or arachidonoyl-CoA was the substrate. The latter findings were confirmed in isolated glomeruli (Table 3). Malondialdehyde, an end product of lipid peroxidation, was significantly lower in the renal cortex of diabetic rats compared to that of control rats (0.63 ± 0.02 *vs.* 0.77 ± 0.02 nmol/mg protein, n = 17, $P < 0.01$). In isolated glomeruli of diabetic rats, malondialdehyde was not significantly different from that of controls (1.05 ± 0.07 *vs.* 0.98 ± 0.06 nmol/mg protein, n = 6, $P > 0.2$).

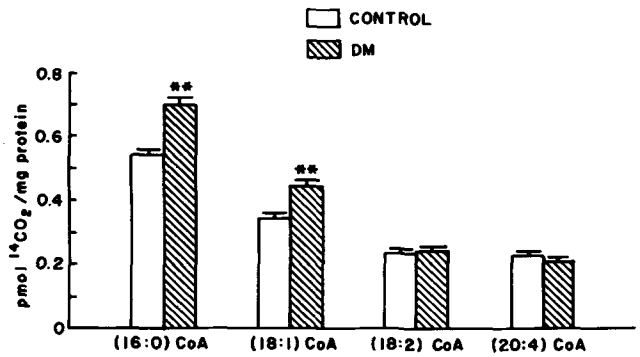

FIG. 1. β-Oxidation rates of palmitoyl-CoA, oleoyl-CoA, linoleoyl-CoA and arachidonoyl-CoA by acyl-CoA oxidase in renal cortical homogenate of control and diabetic rats. Bars denote mean ± SE, n = 17. Asterisks denote that data are significantly different from control, $P < 0.01$.

Basal PGE_2 production by glomeruli of diabetic rats was significantly greater than that of control glomeruli (1,423 ± 79 *vs.* 1,002 ± 44 pg/mg protein, n = 12, $P < 0.01$). The calcium ionophore A23187 augmented PGE_2 production to 1,960 ± 91 pg/mg protein in glomeruli of diabetic rats ($P < 0.01$) and to 1,371 ± 66 pg/mg protein in glomeruli of control rats ($P < 0.01$). The fractional increase of PGE_2 in response to A23187 was 38% in glomeruli of diabetic rats and 37% in glomeruli of control rats. Basal PGE_2 production by isolated renal cortical tubules of diabetic rats (125 ± 15 pg/mg protein) was not different from that of control rats (130 ± 7 pg/mg protein, $P > 0.2$). These values are less than 10 percent of the respective basal glomerular PGE_2 synthesis. Furthermore, no increased PGE_2 production was detected in isolated tubules in response to A23187 (data not shown).

DISCUSSION

This study confirms our previous observation (14) that the amount of arachidonic acid esterified in renal cortical lipids of rats with streptozotocin-induced diabetes mellitus is greatly reduced, and that the reduction of arachidonic acid is accompanied by significant increases of precursor fatty acids involved in the arachidonic acid synthetic pathway. The present study extends our earlier observation by demonstrating that the reduction of esterified arachidonic acid occurs both in renal cortical tubular cells and in renal glomerular cells. The latter observation in glomeruli confirms the earlier report of Clark *et al.* (8). We have also confirmed the work of other investigators who showed that the depression of esterified arachidonic acid occurs in the liver (1-5) and in the heart (5,10) of diabetic rats. Thus, our results add to a growing body of data derived from human and animal studies (1-13), indicating that diabetes mellitus is complicated by a general disturbance of arachidonic acid metabolism and is characterized by a depression of arachidonic acid esterified in the lipids of most, if not all, organs and tissues.

The main objective of our study was to elucidate the major mechanism(s) responsible for the depression of

arachidonic acid in the renal cortex of diabetic rats. We investigated, therefore, the possible roles of decreased synthesis and increased consumption of arachidonic acid. Linoleic acid, the starting substrate for the synthesis of arachidonic acid, is not synthesized in mammalian cells and must be supplied by dietary intake. The observation that linoleic acid was higher in tissues from diabetic rats than in tissues from control rats eliminates the possibility that the low level of arachidonic acid is a consequence of an inadequate supply of substrate. The data are consistent with decreased utilization of linoleic acid for the synthesis of arachidonic acid. Strong support for this conclusion is provided by the data on the activities of the five enzymes involved in the synthesis of arachidonic acid from linoleic acid and the subsequent esterification of arachidonic acid in phospholipids. Of the five enzymes involved in these processes, we found that the activities of two enzymes, Δ6 and Δ5 desaturase, were significantly depressed, whereas the activities of the three remaining enzymes, acyl-CoA synthetase, γ-linolenoyl-CoA elongase and 1-acyl-GPCAT, were either increased or unchanged in renal cortex and in glomeruli of diabetic rats. Decreased activities of Δ6 and Δ5 desaturases were also found in liver and in myocardium of diabetic rats. Thus, our results are internally consistent in that the renal cortex, the liver and the heart exhibited depressed levels of arachidonic acid esterified in lipids and also exhibited depressed activities of Δ6 and Δ5 desaturases. These data, taken together with the evidence that arachidonic acid consumption in the renal cortex of diabetic rats was not appreciably increased *(vide infra)*, provide strong support for the conclusion that the depression of arachidonic acid is caused almost entirely by decreased synthesis.

Decreased Δ5 desaturase activity appears to be more important than decreased Δ6 desaturase in causing decreased synthesis of arachidonic acid (at least in the renal cortex and glomeruli of diabetic rats). Dihomo-γ-linolenic acid, the substrate for Δ5 desaturase, was increased in these tissues. This finding implicates decreased enzyme activity rather than inadequate substrate availability as the mechanism responsible for decreased synthesis of arachidonic acid. By contrast, γ-linolenic acid, the product generated by the action of Δ6 desaturase on linoleic acid, was increased in renal cortex and in glomeruli of diabetic rats despite the fact that Δ6 desaturase activity was depressed. This finding implies that the conversion of linoleic acid to γ-linolenic acid was not the rate limiting step for arachidonic acid synthesis in these tissues. Δ5 Desaturase activity is probably also the major factor responsible for decreased synthesis of arachidonic acid in the heart of diabetic rats in view of the findings that the changes in Δ5 and Δ6 desaturase activities and in fatty acid composition of lipids in the heart were directionally similar to those observed in the renal cortex. Because no significant differences were detected between the levels of γ-linolenic acid or between the levels of dihomo-γ-linolenic acid in the livers of control and diabetic rats, no conclusion can be drawn concerning the relative importance of decreased Δ5 and Δ6 desaturase activities in the depression of arachidonic acid synthesis in the liver of diabetic rats.

Our enzyme data are in agreement with the results of other investigators who have demonstrated decreased activities of Δ6 and Δ5 desaturase in various organs and tissues of diabetic animals (1,4,15–19). By contrast, in the only study of desaturase activity in the kidney of diabetic rats, Clark and Queener (20) reported that Δ6 desaturase pathway activity was increased in renal cortex but was unchanged in liver of alloxan-induced diabetic rats. Although no data were provided, the authors stated that they obtained similar results in rats with streptozotocin-induced diabetes mellitus. The reason for the discrepancy between the results of Clark and Queener (20) and our study on Δ6 desaturase activity in the renal cortex and between the results of Clark and Queener (20) and those of other investigators (4,15,16,18) (including our own) on Δ6 desaturase activity in the liver most likely relates to significant differences in methodology. Clark and Queener (20) used unesterified arachidonic acid or linoleic acid as substrate and added CoASH, whereas we used the CoA esters of these fatty acids as substrates. Clark and Queener (20) also used a crude enzyme preparation that included soluble factors, whereas we used a traditional microsomal preparation. The investigators showed that the desaturase pathway activity in their experiments was influenced by long chain acyl-CoA synthetase activity as they detected no difference between the Δ6 desaturase pathway activities in renal cortex of normal and diabetic rats when exogenous long-chain acyl-CoA synthetase was included in the assay reaction. Clark and Queener (20) suggested that previous studies in which Δ6 desaturase activity was reported to be depressed were open to question because the investigators had failed to document that the enzymatic reaction was linear under the assay conditions employed. That criticism, however, does not pertain to our study.

No disagreement exists with respect to our findings of increased activities of linoleoyl-CoA synthetase and of arachidonoyl-CoA synthetase in renal cortex and in glomeruli of diabetic rats, as similar results were obtained for the renal cortex of diabetic rats by Clark and Queener (20) and in glomeruli of diabetic rats by Kanzaki *et al.* (22). Our observation that 1-acyl-GPCAT activity was increased in renal cortex and in glomeruli of diabetic rats is in agreement with similar findings in glomeruli of diabetic rats by Kanzaki *et al.* (22) and in liver of diabetic rats by Dang *et al.* (28).

Insulin-dependent diabetes mellitus is complicated by a shift in metabolism from carbohydrates to fatty acids as a source of fuel (31,32). Moreover, it is well established that renal proximal tubular cells have the capacity to transport and metabolize fatty acids (33,34). Therefore, we assessed the possibility that increased β-oxidation of fatty acids might contribute to the pathogenesis of low arachidonate levels in the renal cortex of diabetic rats. Although β-oxidation of palmitoyl-CoA and of oleoyl-CoA was significantly higher in the renal cortex of diabetic rats compared to that of control rats, no difference was detected between the acyl-CoA oxidase activities of renal cortex or glomeruli of diabetic and control rats when linoleoyl-CoA and arachidonyl-CoA were the substrates. Thus, increased consumption of arachidonic acid or its precursor by β-oxidation cannot be implicated as a cause of the reduced level of arachidonic acid in the renal cortex or in glomeruli of diabetic rats.

Arachidonic acid is the most abundant polyunsaturated fatty acid in mammalian tissue and is highly susceptible to free radical attack (35,36). Increased lipid peroxidation and increased activities of enzymes involved in peroxide

metabolism have been observed in diabetes mellitus (37). In the renal cortex, peroxisomal β-oxidation is stimulated in diabetes mellitus (32) and this metabolic pathway generates hydroxyl radicals (38,39). Therefore, we considered the possibility that consumption secondary to increased lipid peroxidation might contribute to the depression of arachidonic acid in the renal cortex of diabetic rats. For this purpose we measured malondialdehyde, an end product of lipid peroxidation (35). However, the level of malondialdehyde in the renal cortex of diabetic rats was significantly lower than that of control rats, a finding more consistent with a reduced rate of lipid peroxidation, possibly due to the reduced quantity of arachidonic acid present.

Finally, we considered the possibility that increased consumption of arachidonic acid in support of eicosanoid synthesis might contribute to the depression of this fatty acid in the renal cortex of diabetic rats. In agreement with the results of other investigators (40), we found that basal and stimulated PGE_2 synthesis were higher in glomeruli from diabetic rats when compared to PGE_2 synthesis in glomeruli from control rats. Thus, increased consumption of arachidonic acid to support prostaglandin synthesis may contribute to the depression of arachidonic acid in glomeruli of diabetic rats. In renal cortical tubules, however, basal PGE_2 synthesis was similarly low in both groups and did not increase in response to stimulation with A23187. It remains uncertain whether the trivial level of PGE_2 synthesis in renal cortical tubules reflects the presence of contaminating glomeruli or is derived from cells of the cortical collecting tubule. As tubules comprise the vast bulk of the renal cortex, the low rate of PGE_2 synthesis detected in renal cortical tubules argues against increased consumption of arachidonic acid in support of prostaglandin synthesis as a significant factor contributing to the depression of arachidonic acid.

Arachidonic acid may also be metabolized by the lipoxygenase pathway which, in the kidney, is found primarily in glomeruli (40,41), with little or no activity detectable in renal cortical tubules (41–43). Arachidonic acid can also be metabolized by cytochrome P-450 dependent monooxygenases (44,45) and this enzyme system is present in renal proximal tubules. We did not measure the metabolism of arachidonic acid by the lipoxygenase pathway and the P-450 pathway in our study, and no information is available in the literature concerning the activities of these pathways in the renal cortex of diabetic rats. Although unlikely to be a major factor, we cannot exclude the possibility that increased activity of these pathways may contribute to the depression of arachidonic acid in the renal cortex of diabetic rats.

In summary, the results of our experiments support the conclusion that the depression of arachidonic acid esterified in lipids of the renal cortex and glomeruli of rats with diabetes mellitus is principally due to decreased synthesis of arachidonic acid consequent to depression of Δ6 and Δ5 desaturases. No evidence was found to implicate increased consumption of arachidonic acid secondary to accelerated lipid peroxidation or to β-oxidation. Increased consumption of arachidonic acid to support prostaglandin synthesis may contribute to the depression of arachidonic acid in glomeruli, but not in renal cortical tubules of diabetic rats. The possible role of increased consumption of arachidonic acid by the lipoxygenase and cytochrome P-450 pathways remains to be assessed.

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Effect of High Fat Corn Oil, Olive Oil and Fish Oil on Phospholipid Fatty Acid Composition in Male F344 Rats

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Epidemiological and laboratory animal model studies have provided evidence that the effect of dietary fat on colon tumorigenesis depends on the amount of fat and its composition. Because of the importance of the composition of dietary fat and of tissue membrane fatty acid composition in tumor promotion, experiments were designed to investigate the relative effects of high fat diets rich in ω3, ω6 and ω9 fatty acids and colon carcinogen on the phospholipid fatty acid composition of liver, colon, small intestine, erythrocytes and blood plasma. At 6 wk of age, groups of animals were fed diets containing 5% corn oil (LFCO), 23.5% corn oil (HFCO), 23.5% olive oil (HFOO), and 20.5% fish oil plus 3% corn oil (HFFO). Two weeks later all the animals except the vehicle-treated animals received azoxymethane s.c. once weekly for 2 wk at a dose rate of 15 mg/kg body weight. Animals were sacrificed 5 d later and liver, colon, small intestine and erythrocytes and blood plasma were analyzed for phospholipid fatty acids. The results indicate that the phospholipid fatty acid composition of liver, colon and small intestine of HFCO diet fed animals, were not significantly different from those fed the LFCO diet. The levels of palmitoleic acid and linoleic acid were increased in erythrocytes and blood plasma of the animals fed the HFCO diet compared to those fed the LFCO diet. Feeding the HFOO diet significantly increased the oleic acid content and decreased the linoleic acid and arachidonic acid levels in various organs when compared to the HFCO diet. Animals fed the HFFO diet showed a marked increase in eicosapentaenoic acid and docosahexaenoic acid and a decrease in linoleic acid and arachidonic acid levels as compared to those fed the HFCO diet. The results also indicate that carcinogen treatment had only a minimal effect on the phospholipid fatty acid composition.
Lipids 28, 441–447 (1993).

Several epidemiological studies have demonstrated that diets particularly high in total fat generally correlate with an increased risk for the development of colon cancer in humans (1–4). Laboratory animal model studies have provided evidence that the effect of dietary fat on colon tumorigenesis depends on the amount and types of fat consumed. For example, high fat diets containing corn oil, safflower oil, beef fat or lard increased the chemically-induced colon tumors when compared to the respective low fat diets, whereas the diets containing high levels of coconut oil, olive oil or fish oil had no effect on colon tumor promotion (4–10). One study showed that even at a low dietary fat level, rats fed the diet containing 5% linoleate showed a greater tumorigenesis than did rats fed 0.3% linoleate and 4.7% stearate (7). These studies suggest that the differential effect of various high fat diets on colon tumorigenesis, at least in part, depends on the fatty acid composition of the diets consumed.

Dietary lipids have been shown to induce extensive modification in the fatty acid composition of cell membranes which in turn affects various cellular functions (11–14). Modifications in the fatty acid composition can influence the activities of various membrane-bound enzymes and carriers (15,16). For example, phospholipids composed of mainly essential fatty acids are required for optimal mixed function oxidase activities (17). Diets containing corn oil increase the mixed function oxidase activity and cytochrome P_{450} levels in rat liver (18). Many carcinogens require metabolic activation in liver and other organs, including colon, to exert their carcinogenic effect (19). These activating enzymes have been shown to be influenced by dietary fats (18,20). In addition, several xenobiotics and other environmental and dietary carcinogens alter the lipid metabolism through modulation of these enzymes (20,21).

In view of significance of both amount and types of dietary fat and membrane fatty acid composition in tumor promotion, experiments were designed to assess the relative effect of high fat diets rich in ω3 (fish oil), ω6 (corn oil) and ω9 (olive oil) fatty acids on phospholipid fatty acid composition of the liver, colon, small intestine, erythrocytes and plasma. Also the effect of carcinogen on the phospholipid fatty acid composition of these organs was investigated.

MATERIALS AND METHODS

Animals, diets and carcinogen. Weanling male F344 rats were purchased from Charles River Breeding Laboratories (Kingston, NY). Cold pressed extra virgin olive oil was obtained from a supermarket. Fish oil was supplied by the National Institutes of Health through the National Marine Fisheries Service (Charleston, SC). The ingredients of semipurified diets were purchased from Dyets, Inc. (Bethlehem, PA) and azoxymethane (AOM) was from Ash-Stevens (Detroit, MI). A total of 64 male F344 rats received at weaning were quarantined for 7 d and then randomly assigned to one of four dietary groups of 16 animals each. Each dietary group was then divided equally into vehicle-treated and AOM-treated subgroups. They were housed three to a plastic cage with filter tops and maintained under controlled conditions of 21°C, 50% humidity and 12-h light/dark cycle. All animals were fed *ad libitum*. The food cups were replenished every day.

The compositions of the experimental diets were based on modified AIN-76A diets (22,23) and are shown in Table 1. The percent composition of all experimental diets was adjusted so that the animals in all dietary groups would consume the same amount of calories, protein, vitamins, minerals and fiber. All the diets were prepared in our laboratory three times weekly and stored in a cold room at 4°C in the dark in air-tight containers filled with nitrogen. Aliquots of experimental diets were analyzed for their fatty acid composition.

Experimental procedure. Beginning at 5 wk of age, all animals were fed the control diet containing 5% low fat corn oil (LFCO). At 6 wk of age, *i.e.*, two weeks before AOM

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Abbreviations: AOM, azoxymethane; HFCO, high fat corn oil diet; HFOO, high fat olive oil diet; HFFO, high fat fish oil diet; HPLC, high-performance liquid chromatography; LFCO, low fat corn oil diet.

TABLE 1

Composition of Experimental Diets[a]

Diet ingredients	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
		(g/kg diet)		
Casein	200	235	235	235
DL-Methione	3.0	3.5	3.5	3.5
Corn starch	520	329	329	329
Dextrose	130	83.2	83.2	83.2
Alphacel	50	59	59	59
Corn oil	50	235	0	30
Olive oil	0	0	235	0
Fish oil	0	0	0	205
Mineral mix	35	41.1	41.1	41.1
Vitamin mix	10	11.8	11.8	11.8
Choline bitartrate	2.0	2.4	2.4	2.4

[a]This diet was prepared on the basis of the American Institute of Nutrition standard reference diet with the modification of varying sources of carbohydrate.

[b]LFCO, low fat corn oil diet; HFCO, high fat corn oil diet; HFOO high fat olive oil diet; HFFO, high fat fish oil diet. Additional amounts of corn oil or olive oil and fish oil were added at the expense of starch and dextrose. The composition of high fat diets was adjusted so that all animals in various dietary groups would consume approximately the same amount of protein, minerals, vitamins, fiber and calories.

TABLE 2

Fatty Acid Composition of Experimental Diets[a]

Fatty acid	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0.1	0.4	ND
14:0	0.4	0.5	6.7
16:0	11.2	12	18.2
16:1n-7	0.2	1.5	7.2
18:0	2.0	2.2	2.7
18:1n-9	24.3	72.1	15.9
18:2n-6	58.8	9.3	7.1
18:3n-3	1.2	0.2	0.9
20:4n-6	0	0	1.2
20:5n-3	0	0	17.4
22:6n-3	0	0	13.1
Saturated fatty acids	13.7	15.3	27.9
n-3	1.2	0.2	30.9
n-6	58.8	9.3	8.3
n-9	24.3	72.1	15.9

[a]Mean percentage composition of fatty acids (n = 2).

[b]HFCO, high fat corn oil diet; HFOO, high fat olive oil diet; HFFO, high fat fish oil diet.

or saline (vehicle) treatment, groups of animals were placed on experimental diets containing 5% low fat corn oil (LFCO), 23.5% high fat corn oil (HFCO), 23.5% high fat olive oil (HFOO) or 20.5% high fat fish oil plus 3% corn oil (HFFO). At the beginning of 8 and 9 wk of age, all animals except the vehicle-treated animals received AOM subcutaneously once weekly at a dose rate of 15/mg/kg body wt/wk. Five days after the last AOM injection, all the animals were bled by heart puncture under CO_2 anesthesia. They were then killed and livers were excised and frozen in liquid nitrogen. The colon and small intestine were rapidly removed and rinsed in ice-cold normal saline. They were slit open longitudinally and freed from all contents. They were laid flat on a glass plate, and mucosa scraped with a microscopic glass slide. The samples were quickly frozen in liquid nitrogen and stored at −80°C.

Analytical methods. Blood samples were centrifuged at 3,000 × *g* for 10 min at 4°C to separate plasma from cells. Erythrocyte ghosts were obtained according to the method described by Hanahan and Ekholm (24). Liver and colonic and small intestinal mucosal samples were homogenized in 0.15 M KCl using a polytron homogenizer. Debris and nuclei were removed by centrifugation at 1500 × *g* for 10 min at 4°C. Microsomes were pelleted by centrifuging the supernatant at 105,000 × *g* for 1 h at 4°C. Methods for isolation of phospholipids from erythrocytes, plasma and from microsomal fractions of liver, colon and small intestine were as described by Bligh and Dyer (25) using chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene (0.005%). Fatty acid phenacyl esters from phospholipids were prepared according to the method of Borch (26). The fatty acid derivatives were analyzed by high-performance liquid chromatography (HPLC) using a Waters HPLC system driven by model 510 system controller (Waters Associates, Milford, MA). Briefly, the fatty acid derivatives were separated on a Waters μ Bond pack C_{18} column (30 cm length × 4 mm diameter) using a step-wise gradient of acetonitrile and water at a flow rate of 1.5 mL/min. Elutions of fatty acid phenacyl esters were monitored at 240 nm wavelength in a Waters 990 Photodiode Array Detector (Waters Associates). Individual fatty acids were identified by comparing to authentic standards derivatized and analyzed under the same conditions and by the comparison of retention times.

Statistical analysis. Data are presented as means ± SD. Significant difference in values of fatty acids between various dietary groups was analyzed by analysis of variance and Student *t*-test. The data were analyzed routinely using both the log transformed and arithmetic values of the dependent variables (in the case of percentage composition of fatty acids) in view of the assumption of normality proscribed by parametric tests, such as the *t*-test. When the results were similar, we assumed that there was no significant departure from normality and reported only the *P*-values based on the arithmetic comparison. Furthermore, our *P*-values were based on the Student's *t*-distribution rather than normal distribution to further account for deviations from the standard normal curve.

The conventional method of adjusting for the critical *P*-values (α error) is by dividing the usual probability (*e.g.*, $P = 0.05$) by the number of comparisons made (α/k). Thus, while the *P*-values listed in the tables that are below 0.01 remain significant even after adjustment, those values between 0.05 and 0.01 lose their significance and should, therefore, be interpreted with caution. The potential interactions between the diet and carcinogen with regard to fatty acid composition have been evaluated using a factorial analysis of variance model with diet, carcinogen and diet *vs.* carcinogen interactions.

RESULTS

The fatty acid composition of the experimental diets is summarized in Table 2. As expected, corn oil, olive oil and fish oil diets contain high levels of linoleic acid, oleic acid and ω3 fatty acids (eicosapentaenoic acid and docosa-

hexaenoic acid), respectively. The body weights and the weights of colonic and small intestinal mucosa and liver were similar among the dietary groups (Table 3).

The fatty acid composition of plasma phospholipids in animals fed various experimental diets and treated with AOM is shown in Table 4. In animals fed the HFCO, a slight but significant increase in the plasma palmitoleic acid, linoleic acid and eicosapentaenoic acid were observed when compared to those fed the LFCO diet. When compared to HFCO, HFOO-fed animals showed a significant increase in the plasma oleic acid and a decrease in linoleic acid. Feeding of HFCO or HFOO diets had no measurable effect on the palmitic acid and stearic acid content of plasma. The plasma eicosapentaenoic acid and docosahexanoic acid were significantly increased, and stearic, linoleic and arachidonic (20:4n-6) acids were decreased in the animals fed the HFFO diet compared to those fed the HFCO diet. The results also indicate that carcinogen treatment did not significantly alter the plasma fatty acid composition irrespective of the type of fat in the diet.

The fatty acid composition of erythrocyte phospholipids is shown in Table 5. Dietary HFCO caused a slight increase in palmitoleic acid and linoleic acid as compared to LFCO. Oleic acid was significantly higher and stearic acid, linoleic acid and arachidonic acids were lower in animals fed the HFOO diet as compared to those fed the HFCO diet. Animals fed the HFFO diet showed a marked increase in eicosapentaenoic acid and docosahexaenoic acid, the two major ω3 fatty acids, and a signifi-

TABLE 3

Effect of Experimental Diets on the Body, Colonic Muscosal, Small Intestinal Mucosal and Liver Weights[a]

Dietary group[b]	Body weight (g)	Colonic mucosal weight (mg)	Small intestinal mucosal weight (g)	Liver weight (g)
Saline				
LFCO	255 ± 14.5	326 ± 15	1.7 ± 0.1	10.5 ± 0.6
HFCO	242 ± 9.8	328 ± 23	2.1 ± 0.2	9.48 ± 0.8
HFOO	243 ± 10.2	335 ± 31	1.9 ± 0.2	9.26 ± 0.7
HFFO	238 ± 12.1	340 ± 24	1.7 ± 0.2	10.8 ± 0.8
AOM[c]				
LFCO	248 ± 12.0	350 ± 30	1.8 ± 0.1	9.83 ± 0.6
HFCO	257 ± 13.3	388 ± 20	1.8 ± 0.1	10.8 ± 0.7
HFOO	252 ± 14.6	395 ± 17	1.7 ± 0.2	10.2 ± 0.9
HFFO	243 ± 13.2	374 ± 19	1.6 ± 0.2	11.0 ± 0.7

[a]Values are means ± SE (n = 8).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]AOM, azoxymethane.

TABLE 4

Percentage Composition of Fatty Acids in the Plasma Phospholipids[a]

Fatty acid	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1[e,h]
14:0	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.8 ± 0.1[g]
16:0	18.4 ± 2.4	19.6 ± 2.1	19.3 ± 1.7	23.4 ± 2.7[f]
16:1n-7	0	0.8 ± 0.2	1.3 ± 0.2[e,f]	1.6 ± 0.4[g]
18:0	17.9 ± 1.3	18.4 ± 1.6	18.4 ± 2.4	14.5 ± 1.3[g]
18:1n-9	7.5 ± 0.8	10.5 ± 0.8[d,f]	21.5 ± 1.6[i]	8.8 ± 0.8[f]
18:2n-6	20.4 ± 1.8	23.9 ± 2.2[f]	11.8 ± 0.8[i]	7.6 ± 1.5[i]
18:3n-3	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1[f]	0.5 ± 0.1[g]
20:4n-6	20.8 ± 2.1	19.2 ± 1.7	18.6 ± 1.4	11.3 ± 1.4[i]
20:5n-3	0	0.26 ± 0.1	0.6 ± 0.2[f]	10.7 ± 1.3[i]
22:6n-3	2.8 ± 0.5	3.1 ± 0.4	2.9 ± 0.6	9.3 ± 1.4[i]
SFA[c]	36.8	38.5	38.2	38.9
n-3	3.1	3.7	4.0	20.4
n-6	41.2	43.1	30.4	18.9
n-9	7.5	10.5	21.5	8.8
Total	88.6	89.5	95.5	88.6

[a]Values are means ± SD (n = 6); animals were treated with azoxymethane (AOM).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]SFA, saturated fatty acids.
[d]Significantly different from animals fed LFCO and treated with AOM; [f]$P < 0.05$, [g]$P < 0.01$.
[e]Significantly different from animals fed HFCO and treated with AOM: [f]$P < 0.05$, [g]$P < 0.01$, [h]$P < 0.001$, [i]$P < 0.0001$.

cant decrease of stearic acid, linoleic acid and arachidonic acid, as compared to those fed the HFCO diet. AOM treatment had a minimal effect on fatty acid composition of erythrocyte phospholipids.

The percentage composition of fatty acids of colon and small intestine and liver phospholipids are shown in Tables 6, 7 and 8, respectively. Dietary HFCO had no effect on the levels of fatty acids of colon, small intestine and liver as compared to the LFCO group. In the olive oil (HFOO) group, there was a marked increase in oleic acid and a slight increase in ω3 fatty acids and a decrease in linoleic acid and arachidonic acid content of all three organs. In contrast, the HFFO diet increased the eicosapentaenoic acid and docosahexaenoic acid levels and decreased the oleic acid, linoleic acid and arachidonic acid in the colon, small intestine and liver. AOM-treatment had no effect on the fatty acid profile of all three organs.

DISCUSSION

The results of the present study are of considerable interest because they demonstrate that the types of fat in

TABLE 5

Percentage Composition of Fatty Acids in the Erythrocyte Phospholipids[a]

Fatty acid	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1[e,h]
14:0	0.9 ± 0.2	1.1 ± 0.2	1.2 ± 0.3	1.7 ± 0.4[f]
16:0	30.4 ± 3.1	33.1 ± 3.9	28.4 ± 2.5	30.2 ± 3.2
16:1n-7	0.1 ± 0.1	1.3 ± 0.2	1.8 ± 0.2[e,g]	1.7 ± 0.6
18:0	15.6 ± 1.5	14.8 ± 1.3	13.8 ± 2.1	9.1 ± 0.8[i]
18:1n-9	10.7 ± 1.6	11.5 ± 1.1	17.3 ± 1.2[i]	12.4 ± 1.1
18:2n-6	11.3 ± 1.3	14.2 ± 1.3[d,g]	10.5 ± 1.1[h]	6.5 ± 0.7[i]
18:3n-3	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.1[f]	0.6 ± 0.1
20:4n-6	18.2 ± 2.7	17.2 ± 1.8	16.4 ± 1.1	11.0 ± 1.2[i]
20:5n-3	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	7.1 ± 0.1[i]
22:6n-3	2.8 ± 0.4	2.9 ± 0.6	2.6 ± 0.7	8.5 ± 1.0[i]
SFA[c]	47.0	49.2	43.5	41.6
n-3	3.5	3.7	3.7	16.3
n-6	29.5	31.6	26.9	17.5
n-9	20.7	11.5	17.3	12.4
Total	90.9	97.3	93.5	89.5

[a]Values are means ± SD (n = 6).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]SFA, saturated fatty acids.
[d]Significantly different from animals fed LFCO and treated with azoxymethane (AOM): [f]$P < 0.05$, [g]$P < 0.01$, [h]$P < 0.001$.
[e]Significantly different from animals fed HFCO and treated with AOM: [f]$P < 0.05$, [g]$P < 0.01$, [h]$P < 0.001$, [i]$P < 0.0001$.

TABLE 6

Percentage Composition of Fatty Acids in the Colonic Mucosal Phospholipids[a]

Fatty acid	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1[d,f]
14:0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.2[f]
16:0	13.8 ± 2.3	14.3 ± 2.5	16.1 ± 2.5	15.9 ± 3.7
16:1n-7	2.1 ± 0.4	1.8 ± 0.3	3.3 ± 0.4[d,e]	1.9 ± 0.5
18:0	19.3 ± 4.3	22.1 ± 3.8	17.9 ± 3.1	16.2 ± 2.9[e]
18:1n-9	10.8 ± 1.3	10.4 ± 1.2	20.4 ± 2.1[h]	13.1 ± 1.9[e]
18:2n-6	25.6 ± 3.8	27.5 ± 4.1	10.1 ± 1.2[g]	8.4 ± 1.4[h]
18:3n-3	0.1 ± 0.0	0.1 ± 0.04	0.8 ± 0.4[g]	1.3 ± 0.3[h]
20:4n-6	16.3 ± 2.1	17.1 ± 3.2	12.9 ± 2.1[e]	9.1 ± 1.1[h]
20:5n-3	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	8.2 ± 0.9[h]
22:6n-3	1.3 ± 0.1	1.3 ± 0.1	2.8 ± 0.5[g]	10.1 ± 1.3[h]
SFA[c]	33.3	36.7	34.3	33.0
n-3	1.5	1.7	4.1	19.6
n-6	41.9	44.6	23	17.5
n-9	10.8	10.4	20.4	13.1
Total	89.8	95.3	85.3	85.2

[a]Values are means ± SD (n = 6); and animals treated with azoxymethane (AOM).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]SFA, saturated fatty acids.
[d]Significantly different from animals fed LFCO and treated with azoxymethane (AOM). [e]$P < 0.05$, [f]$P < 0.01$, [g]$P < 0.001$, [h]$P < 0.0001$.

TABLE 7

Percentage Composition of Fatty Acids in the Small Intestinal Mucosal Phospholipids[a]

Fatty acid	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0	0.4 ± 0.0	0.2 ± 0.1[e,f]	0.3 ± 0.1[e,h]
14:0	0.1 ± 0.1	0.2 ± 0.1[d,f]	0.3 ± 0.1	0.4 ± 0.1[f]
16:0	14.4 ± 2.1	15.0 ± 2.3	16.8 ± 1.6	15.0 ± 2.1
16:1n-7	2.1 ± 0.8	2.1 ± 0.6	3.9 ± 0.7[f]	2.1 ± 0.5
18:0	18.5 ± 3.2	21.7 ± 2.8	20.5 ± 3.1	16.2 ± 1.9[g]
18:1n-9	10.4 ± 1.3	9.7 ± 1.2	22.5 ± 2.5[i]	12.8 ± 0.9[h]
18:2n-6	24.5 ± 2.2	28.6 ± 3.7[d,g]	9.2 ± 0.7[i]	8.8 ± 0.8[i]
18:3n-3	0.1 ± 0.1	0.1 ± 0.0	0.8 ± 0.2[h]	1.5 ± 0.2[i]
20:4n-6	17.4 ± 2.2	17.8 ± 2.5	11.3 ± 1.8[i]	8.7 ± 0.7[i]
20:5n-3	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1[f]	9.4 ± 1.1[i]
22:6n-3	1.2 ± 0.1	1.3 ± 0.1	2.5 ± 0.5[i]	11.5 ± 1.3[i]
SFA[c]	33.1	37.0	37.9	32.0
n-3	1.5	1.6	3.7	22.4
n-6	41.9	46.4	20.5	17.5
n-9	10.4	9.7	22.5	12.8
Total	88.8	97.1	88.6	86.9

[a]Values are means ± SD (n = 6) and animals treated with azoxymethane (AOM).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]SFA, saturated fatty acids.
[d]Significantly different from animals fed LFCO and treated with AOM. [f]$P < 0.05$.
[e]Significantly different from animals fed HFCO and treated with AOM: [f]$P < 0.05$, [g]$P < 0.01$, [h]$P < 0.001$, [i]$P < 0.0001$.

TABLE 8

Percentage Composition of Fatty Acids in the Liver Phospholipids[a]

Fatty acid	LFCO[b]	HFCO[b]	HFOO[b]	HFFO[b]
12:0	0	0.1 ± 0[d,f]	0.2 ± 0.1[e,g]	0.3 ± 0.1[e,h]
14:0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1[f]	0.4 ± 0.1[g]
16:0	17.4 ± 1.9	18.7 ± 2.4	14.3 ± 2.1[g]	15.8 ± 1.6
16:1n-7	2.5 ± 0.4	2.3 ± 0.5	4.2 ± 0.5[f]	3.0 ± 0.6
18:0	16.7 ± 1.4	15.5 ± 1.4	14.6 ± 1.7	16.3 ± 1.4
18:1n-9	8.8 ± 0.7	8.5 ± 0.6	20.9 ± 2.1[i]	11.4 ± 0.8[h]
18:2n-6	15.4 ± 2.2	14.6 ± 1.6	8.7 ± 0.7[i]	8.9 ± 1.2[i]
18:3n-3	0.8 ± 0.3	0.3 ± 0.2	0.8 ± 0.2[f]	1.6 ± 0.4[i]
20:4n-6	24.7 ± 2.2	27.4 ± 3.2	17.2 ± 2.2[i]	16.7 ± 1.8[i]
20:5n-3	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	7.7 ± 1.2[i]
22:6n-3	1.4 ± 0.3	1.3 ± 0.4	3.1 ± 0.7[g]	9.4 ± 1.1[i]
SFA[c]	34.3	34.4	29.5	32.8
n-3	2.4	1.9	4.2	18.7
n-6	40.1	42.0	25.9	25.6
n-9	8.8	8.5	20.9	11.4
Total	88.1	89.2	84.72	91.6

[a]Values are means ± SD (n = 6) and animals treated with azoxymethane (AOM).
[b]LFCO, low fat corn oil; HFCO, high fat corn oil; HFOO, high fat olive oil, HFFO, high fat fish oil.
[c]SFA, saturated fatty acids.
[d]Significantly different from animals fed LFCO and treated with AOM. [f]$P < 0.05$.
[e]Significantly different from animals fed HFCO and treated with AOM: [f]$P < 0.05$, [g]$P < 0.01$, [h]$P < 0.001$, [i]$P < 0.0001$.

the diet affect the fatty acid composition of blood plasma, and of the phospholipids of erythrocytes, liver, colon and small intestine in rats. In general, high dietary olive oil increased the oleic acid content of plasma, erythrocytes, liver and intestines and decreased the linoleic acid content of these tissues and of plasma. These results are in agreement with published reports from other laboratories (27-29). Furthermore, in the olive oil-fed animals, the arachidonic acid content was decreased in plasma, erythrocytes and other tissue phospholipids. The inverse relationship between the arachidonic acid and oleic acid of phospholipids suggests that increasing the ratio of dietary ω9/ω6 fatty acids enhances the availability of ω9 fatty acids for incorporation into membrane phospholipids relative to ω6 fatty acids. Also the slightly higher content of ω3 fatty acids observed in phospholipids of the olive oil group as compared to the corn oil group may be related to the lower linoleic acid and linolenic acid ratio

in olive oil. It is well known that competition exists between oleate, linoleate and α-linolenate for the Δ6 desaturase enzyme system. Enzyme affinity decreases in the order linolenic acid (ω3), linoleic acid (ω6) and oleic acid (ω9) (30). Thus, the lower linoleate content of olive oil could enhance α-linolenate desaturation, thereby increasing the ω3 fatty acid in phospholipids of olive oil-fed animals.

This study also demonstrates that high dietary fish oil significantly increased the eicosapentaenoic acid and docosahexaenoic acid levels with a concomitant decrease of linoleic acid and arachidonic acid in phospholipids of various organs. These results are in general agreement with those of studies in which decreased levels of linoleic acid and arachidonic acid were found in plasma, platelets, heart, lung and kidney in animals fed cod liver oil or concentrates of other marine oils (12,27,31-33). It has also been reported that dietary fish oil significantly decreases liver, plasma and platelet arachidonic acid levels (11). Previously, our laboratory had shown a dose-related increase in the incorporation of ω3 fatty acids into the microsomes of colonic mucosal cells when feeding increasing levels of menhaden oil to F344 rats (34).

The fatty acid changes that occur following supplementation of various high fat diets appear to be facilitated through competition among individual saturated, monounsaturated, polyunsaturated and highly polyunsaturated fatty acids for the enzyme system that synthesizes long-chain fatty acids through a sequence of elongation and desaturation reactions (35). Although in our study, the fatty acid composition of various organs was not affected by AOM-treatment factors such as modulation of the ongoing dynamic acyl exchange and transfer, and remodeling involved in the modification of membrane lipid fatty acid composition were affected by AOM-treatment (35,36). In this regard we are not aware of any previous studies on the modulating effect of carcinogens on membrane phospholipids. Our recent study indicates that AOM-treatment significantly enhances plasma, liver and colonic mucosal synthetic activity of prostaglandins, such as PGE_2 and 6-keto $PGF_{1\alpha}$, when compared to saline-treated animals fed similar diets (37). This may be due to increased turnover of membrane arachidonic acid as a consequence of AOM-treatment.

The role of diets containing olive oil and diets high in fish in coronary heart disease and cancer is currently of great interest. There is evidence indicating that populations consuming large amounts of olive oil or marine animals have a decreased incidence of heart disease (38-41). Laboratory animal model studies conducted in our laboratory indicated that high dietary olive oil or fish oil had no colon tumor promoting effect when compared to diets high in corn oil or safflower oil (34,42). Similar results were observed with olive oil on the 7,12-dimethylbenz[a]anthracene and *N*-methyl-*N*-nitrosourea induced mammary tumorigenesis (43-45) and fish oil on AOM induced colon tumorigenesis (34,42,46). Recently, Laseken *et al.* (47) showed that animals fed olive oil and treated with 7,12-dimethylbenz[a]anthracene had a longer latency period, fewer tumors per rat and lower tumor incidence as compared to those fed safflower oil or olive oil plus linoleic acid. This may be partly due to an increase in oleic acid content with an associated decrease in linoleic and arachidonic acid in membrane phospholipids. Whether this altered composition of oleic acid and linoleic acid content of phospholipids has any significant effect on tumorigenesis remains to be established. Previously, several studies have shown a positive correlation between the amount of ω3 fatty acids in the microsomes of colon tumors (34) or mammary tumors (48) and the amount in the fish oil diet. Also, the results of earlier studies indicate that the diets containing high levels of beef fat, lard, corn oil or safflower oil increase the incidence of colon tumors when compared to a low fat diet (4-9). Thus, the results of the present study and of a previous investigation (34) suggest that high fat intake is a necessary but not a sufficient condition for colon tumor promotion and that the fatty acid composition of the dietary fat is one of the determinants of the tumor promoting effect of a high fat diet.

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Effect of Varying Proportions of Dietary Menhaden and Corn Oil on Experimental Rat Mammary Tumor Promotion[1]

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Dose-related effects of long-chain highly unsaturated n-3 fatty acids on the development of *N*-nitrosomethylurea (NMU)-induced rat mammary tumors were assessed in female F344 rats. Four test groups (36 rats/group) were fed the following high-fat (HF) diets (23% fat, w/w): Group 1, 18% menhaden oil (MO) and 5% corn oil (CO); Group 2, 11% MO and 11.8% CO; Group 3, 5% MO and 18% CO; Group 4, CO alone. A fifth group, serving as an internal control, was fed a low-fat diet containing 5% CO alone. Experimental diets were begun after initiation with NMU, and the experiment was terminated 31 wk later. Total tumor numbers in the five groups were 28, 16, 32, 26 and 11, respectively, indicating that the promotion phase of NMU-induced carcinogenesis was significantly suppressed only when equal parts of CO and MO (Group 2) were fed or when CO alone was fed at 5% (w/w). At high (Group 1) or low (Group 3) levels of MO, tumor numbers were indistinguishable from the HF CO group (Group 4). The same pattern was observed when assessed in terms of cumulative tumor incidence and multiplicity. However, when expressed in terms of final tumor incidence, dietary MO did not suppress tumor promotion in a statistically significant fashion at any concentration. Animals fed MO gained weight at the same rate as those fed CO, indicating that the presence of MO in the diet did not result in food avoidance behavior. Measurement of total serum cholesterol indicated an inverse trend with respect to the MO content of the diet. Analysis of serum fatty acid profiles indicated that the proportion of n-3 and n-6 polyunsaturated fatty acids (PUFA) in the serum reflected that of the diet. These results support the hypothesis that the relative proportions of dietary n-3/n-6 fatty acids play an important role in the suppression of experimental mammary tumorigenesis and suggest that changes in circulating cholesterol or n-3 PUFA levels, induced by dietary MO, are not directly related to tumor development.
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It is well established that the development of carcinogen-induced, transplantable and virus-induced mammary tumors is enhanced by high-fat (HF) diets (1). In addition, it has been shown that diets containing high levels of the n-6 diunsaturated fatty acid linoleic acid (LA) stimulate the promotion phase of experimental mammary carcinogenesis (2,3) as well as metastasis to distant organs (4,5), whereas diets containing high levels of medium-chain saturated fatty acids (FA) or monounsaturated FA such as oleic acid (18:1n-9), do not (6).

A fourth class of fatty acids, the n-3 polyunsaturated fatty acids (PUFA) has been shown to exhibit protective effects, in most but not all cases, in primary tumor models including mammary (7-19), colon (20,23), prostate (24,25) and pancreas (26), and in one metastasizing mammary tumor model (27). The majority of studies on n-3 PUFA have used menhaden oil (MO) as the source of n-3 PUFA and have focused on mammary cancer. However, many inconsistencies appear in the literature. In some cases, fish oil suppressed tumorigenesis (8,19); while in others it had little effect (7,9). Still in others, fish oil at certain concentrations actually enhanced tumor development (10,17,23). Also of concern is the lack of any consistent dose-response effects of n-3 PUFA on mammary tumorigenesis (8,9,14-16). The reasons for these inconsistencies are unclear but may involve (i) variations in diet composition, (ii) the use of undeodorized MO which reduces diet palatability, (iii) the use of MO, which is LA deficient, as the sole source of fat, (iv) small numbers of animals and (v) differences inherent in the models being used (transplantable *vs.* chemically induced).

The present study was designed to assess whether increasing amounts of dietary n-3 PUFA inhibit the promotion phase of *N*-nitrosomethylurea (NMU)-induced mammary carcinogenesis in a dose-related manner. Three HF groups containing blends of MO and corn oil (CO) were formulated to obtain n-3/n-6 ratios of 1.51, 0.55 and 0.19 (Groups 1-3, respectively). They were compared with HF and low-fat diet (LF) groups (Groups 4 and 5) containing CO only (n-3/n-6 ratio, 0.007) (Fig. 1). To gain insight into possible mechanisms, the effect of feeding these diets on the cholesterol content and FA composition of serum was also examined.

MATERIALS AND METHODS

Experimental protocol. One-hundred and eighty virgin female inbred F344 rats, 28 d of age (Charles River Breeding Laboratories, North Wilmington, MA), were maintained on the standard NIH-07 diet (Zeigler Bros., Gardners, PA) until 50 d of age. All animals were then assigned into groups of 36 animals each by recognized randomization procedures (27) to equalize initial weight. On day 50 of age, all animals received a single dose (37.5 mg/kg body wt) of NMU (CAS:684-93-5) (Ash Stevens Inc., Detroit, MI) by tail vein injection. NMU was dissolved in a few drops of 3% acetic acid and diluted with distilled H_2O to give a stock solution of 10 mg NMU/mL, which was administered within 2 h of formulation (28). At weekly intervals beginning 4 wks after NMU injection, each rat was weighed and the position and date of palpable tumors recorded.

Two days after NMU administration, animals were randomly allocated to one of five different experimental diets. Groups of thirty-six animals each were placed on diets

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Abbreviations: AA, arachidonic acid (20:4n-6); ANOVA, analysis of variance; CO, corn oil; DHA, docosahexaenoic acid (22:6n-3); DMBA, 7,12-dimethylbenz(a)anthracene; EFA, essential fatty acid; EPA, eicosapentaenoic acid (20:5n-3); FA, fatty acid(s); HF, high-fat diet (23% fat, w/w); HIP, hexane/isopropyl alcohol; LF, low-fat diet (5% fat w/w); LA, linoleic acid (18:2n-6); MO, menhaden oil; MMU, *N*-nitrosomethylurea; PUFA, polyunsaturated fatty acid(s); TBHQ, *tert*-butylhydroquinone.

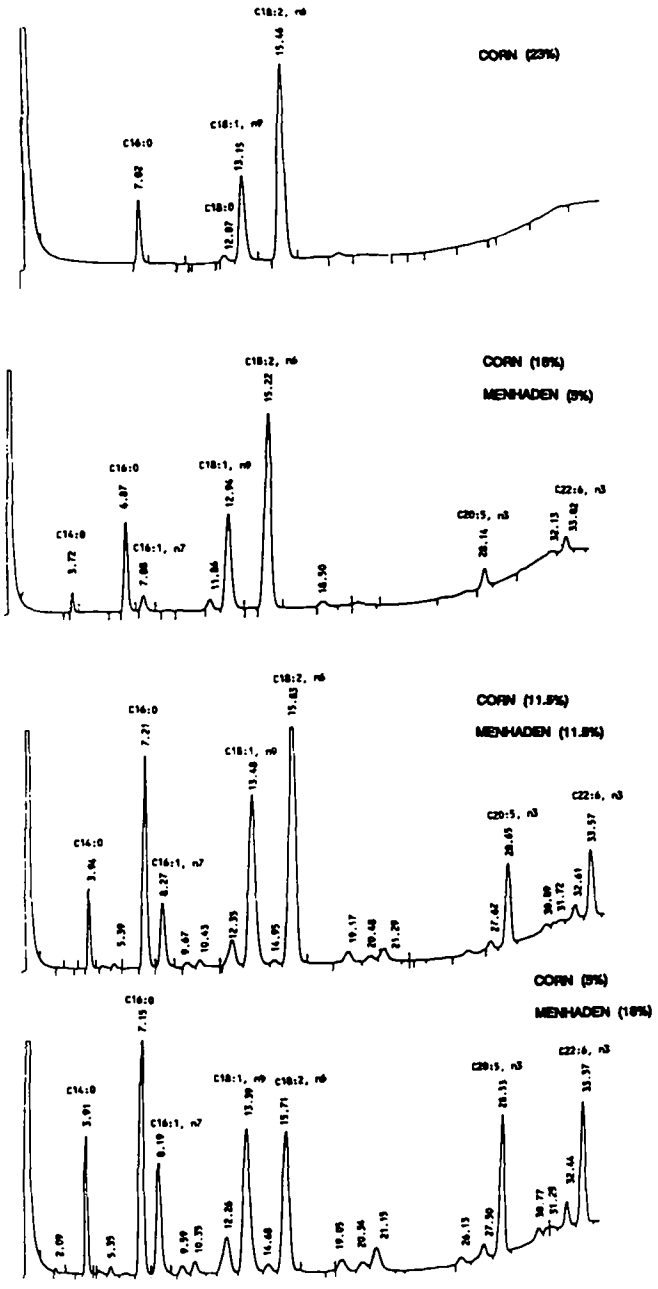

FIG. 1. Comparative fatty acid profiles of corn oil and corn oil/menhaden oil blends. Gas-liquid chromatography (GLC) traces depict the relative area percentages of fatty acids as a function of retention time (numbered vertically). Total lipids were extracted from diets 1-4 and processed for GLC analysis as described in Materials and Methods. Only the major peaks were identified using authentic standards.

containing: 18% MO, 5% CO (Group 1); 11.8% MO, 11.8% CO (Group 2); 5% MO, 18% CO (Group 3); 23% CO (Group 4); and 5% CO (Group 5). The animals remained on the diets for the duration of the experiment (31 wks). Animals were housed three to a polyethylene cage containing hardwood shavings and covered with a filter top. The animal room was controlled for temperature (24 ± 2°C), light (12-h cycle) and humidity (50%). Diets were administered

TABLE 1

Diets[a]

Ingredient	Low-fat diet (g)	Adjusted high-fat diet (g)
Casein	20.0	23.5
Corn starch	52	32.9
Dextrose	13	8.30
Fat	5	23.52
DL-methionine	0.3	0.35
Choline bitartrate	0.2	0.24
Alphacel	5	5.9
(AIN-76) Vitamin mix	1.0	1.18
(AIN-76) Mineral mix	3.5	4.11
Energy value (Kcal/g)	3.89	4.73

[a]High- and low-fat diets based on Newberne *et al.* (31) with replacement of starch/dextrose for sucrose as carbohydrate source.

in powdered form and tap water was provided *ad libitum*. Stainless steel "J" type powder feeders were used to prevent scattering of food. Animals were maintained according to the revised *Guide for the Care and Use of Laboratory Animals* (Department of Health, Education, and Welfare Publications, NIH 85-23, Bethesda, revised 1985) (29).

HF and LF diets. The HF and LF diets used in these experiments were based on the recommendations of the Committee on Laboratory Animal Diets of the National Academy of Sciences (30,31) with slight modifications (Table 1). Because the HF diet is more calorie-dense than the LF diet, less is eaten. The adjusted formulation accounts for this, ensuring that all animals consume the same amount of vitamins, minerals and fiber. The increase in fat in the HF diet was at the expense of starch-dextrose. The HF and LF diets consisted of 23 and 5% fat by weight, respectively. The LF diet was designed to provide approximately 5-6 calories/d of fat, based on an estimated consumption of 45 calories/d (≈12% of total calories). The HF diet provided approximately 20-21 calories/d of fat (≈45% of total calories). The LF and HF diets were designed to mimic the Japanese diet of the 1950s and the U.S. diet during the 1970s (32,33).

Bulk vacuum-deodorized menhaden oil (Lot No. L89195BB) was obtained *gratis* from the Fish Oils Test Materials Program (DOC/NOAA/NIH/ADAMHA) (Southeast Fisheries Center, Charleston, SC). The MO contained 1 g/kg α-tocopherol and 1 g/kg γ-tocopherol and 0.2 g/kg *t*-butyl hydroquinone (TBHQ) as added antioxidants. Food-grade corn oil, provided by BioServ Inc. (Frenchtown, NJ) which contains some α- and γ-tocopherol, was supplemented further with α- and γ-tocopherol and TBHQ to yield an amount of total antioxidants equal to that in MO. Cholesterol was added to the corn oil at 2.0 g cholesterol/kg oil in order to equalize the cholesterol in all five diets.

All dietary ingredients, other than MO, were obtained and diets were prepared by BioServ Inc. In order to prevent the formation of oxidized lipids, diets were formulated and stored undeer the following conditions: both MO and CO were stored under a blanket of N_2 in the dark at 4°C. Batches of diet were mixed and placed in 2- or 4-kg plastic bags, flushed with N_2 and heat-sealed. Upon receipt, aliquots of the diets were rebagged in amounts sufficient for a single feeding per group. The

TABLE 2

Fatty Acid Composition of Corn and Menhaden Oil[a]

Fatty acid	Corn	Menhaden[b]
14:0	—[c]	6.2
16:0	10	15.0
16:1n-7	—	6.5
18:0	2	2.4
18:1n-9	31	8.2
18:2n-6	56	1.2
18:3n-3	0.4	1.0
18:4n-3	—	3.2
20:4n-6	—	0.5
20:4n-3	—	1.4
20:5n-3	—	12.5
22:5n-3	—	1.9
22:6n-3	—	11.0
Total PUFA (%)	56	39.5
Total n-3 (%)	<1	31.9
Total n-6 (%)	56	2.5
n-3/n-6 Ratio	0.007	12.8

[a]Data for corn oil were obtained as previously described (34); data for menhaden oil were provided by Southeast Fisheries Center, Charleston Laboratory. Values are area percentage of total methyl esters.

[b]Percentage of fatty acids adds up to 72%; these represent the major fatty acid species in menhaden oil. In total, menhaden oil contains 11 saturated, 21 monounsaturated, 9 unsaturated and 19 other fatty acids containing from 3 to 6 double bonds.

[c]— = value <0.05%. PUFA, polyunsaturated fatty acids.

bags were flushed with N_2 and heat sealed. All diets were stored at 4°C. Once opened and animals fed, the remaining diet was discarded. Animals were fed three times per week and feeders removed and washed after each feeding.

Quality controls for experimental diets. The fatty acid profile of the MO was provided by the National Marine Fisheries Service (Charleston Laboratory) (Table 2). Fatty acid analyses were conducted in our laboratory as described previously (34). The fatty acid profiles of formulated diets were determined as follows: One gram of diet 1–4 was placed in a pre-weighed 25-mL glass conical test tube, and lipids were extracted with 18 mL of a hexane/isopropyl alcohol (HIP) mixture (3:2). The mixture was then vortexed and the organic layer decanted following centrifugation. After a second extraction with 10 mL HIP, the two organic phases were pooled and evaporated to dryness under a stream of N_2. The conical tube containing the dried residue was weighed to confirm that approximately 0.23 g of lipid was present. Methanolysis was then performed by addition of methanol containing 5% HCl (80°C for 2 h) under N_2. A known amount of pentadecanoic acid was added as an internal standard to the extract prior to methanolysis. The fatty acid methyl esters were extracted with *n*-hexane and quantitatively analyzed by gas-liquid chromatography (Hewlett-Packard 5890, Palo, Alto, CA) using a column packed with 10% DEGS-PS adsorbed on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA). The oven temperature was programmed at 24°C/min from 160 to 225°C. Individual fatty acids were identified by comparison with authentic standard mixtures. All standards were from Nu-Chek-Prep (Elysian, MN). Thiobarbituric acid tests for lipid oxidation products were conducted on formulated diets (35).

Necropsy, serum collection and histopathology. Approximately 31 wk after NMU administration, the experiment was terminated. Blood was drawn by heart puncture under ketamine anaesthesia, collected in evacuated, sterile autosep tubes (Terumo Med., Elkton, MD) and serum separated by centrifugation. Serum was stored at 20°C until use. Animals were then sacrificed by CO_2 euthanasia, and mammary tumors, classified as palpable or nonpalpable but grossly visible, were excised, fixed in 10% buffered formalin, blocked in paraffin and stained with hematoxylin and eosin for histological examination. Histologic diagnosis of mammary tumors was based on criteria outlined by Young and Hallowes (36).

Serum lipid analysis. Total serum cholesterol content in aliquots of serum were determined enzymatically using a DT-60 Clinical Chemistry Analyzer (Kodak Inc., Rochester, NY). The limit of detection of this assay is 30 mg/100 mL and the coefficient of variance is 5%. The method is based on a layered coating dry-slide technique (37,38). Serum fatty acid profiles were determined in the laboratory of Dr. M.T. Clandinin (University of Alberta, Edmonton, Alberta, Canada) using methods described in Reference 39.

Statistical analysis. A tumor-free survival function was estimated separately for each experimental group by the Kaplan-Meier product limit method (40). The survival functions for the different dietary groups were then compared by Gehan's generalized Wilcoxon (41,42). The purpose of this method was to test the null hypothesis that all five survival functions were identical. Differences in the overall frequency of tumor/animal (including tumor-free animals) and in the number of tumors/tumor-bearing animal were assessed by the *Chi* Square test (43). Weight gain over time and the latency of tumors were compared by analysis of variance with multiple comparison procedures (44,45). Trends in serum cholesterol levels as a function of diet were tested by multiple regression analysis (44). Pairwise comparisons of cholesterol levels were performed by analysis of covariance, the covariates being weight gain and latency. The relationship between the presence (or absence) of a tumor and serum cholesterol levels was assessed by the two-tailed *t*-test.

RESULTS

Animal weight gains (Fig. 2) were similar in Groups 1–4, suggesting that differences in dietary MO content did not alter food consumption patterns. It was not possible to measure food consumption accurately because of diet scattering. Group 5, however, exhibited a lower body weight gain when compared to Groups 1–4 which was of borderline significance ($P > 0.10$).

With the exception of mammary tumors, no gross changes in the major organs or organ systems were seen. Mammary tumors were either adenocarcinomas or fibroadenomas, with the fibroadenomas varying in frequency from 0 to 11%. There were 0, 1, 2, 2, 0 unscheduled terminations in Groups 1–5, respectively, due to necrotizing tumors. No animals died of extraneous causes. Tumor incidence data, presented as either total tumor incidence or incidence of adenocarcinoma alone, can be seen in Table 3. Tests for overall trend were nonsignificant for the four HF groups. Pairwise comparisons indicated that Group 1 *vs.* Group 3, Group 2 *vs.* Group 3 and Group 5 *vs.*

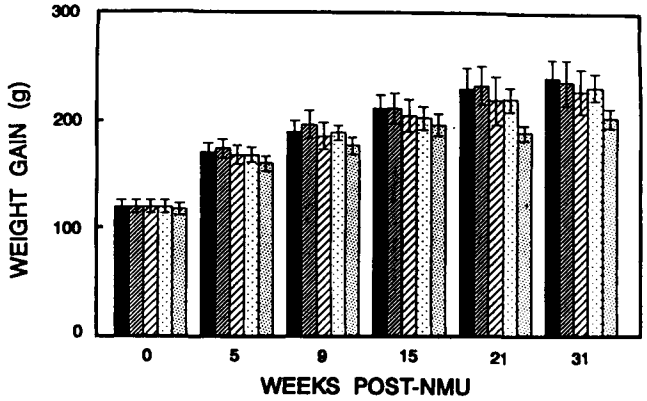

FIG. 2. Mean (± SD) weight gains for Groups 1–5 at selected time points. CO-5/MO-18 Group, solid bars; CO-11.5/MO-11.5 Group, narrow-hatched bars; CO-18/MO-5 Group, broad-hatched bars; CO-23 Group, dotted bars; CO-5 Group, densely dotted bars. Overall comparison is based on analysis of variance, $P < 0.001$. Differences in mean weights for Groups 1–4 *vs.* 5, $P < 0.10$ are by one-tailed *t*-test. Abbreviations: NMU, *N*-nitrosomethylurea; CO, corn oil; MO, menhaden oil.

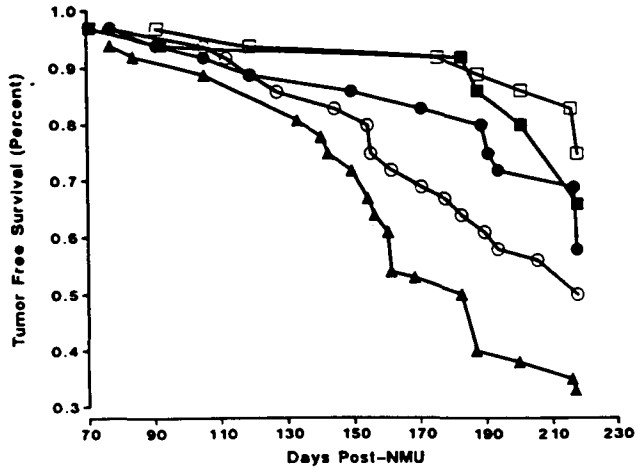

FIG. 3. Kaplan-Meier life table curves for cumulative mammary tumor incidence for Groups 1–5. □, Corn oil (CO) 5%; ■, menhaden oil (MO) 11.5%/CO 5%; ●, MO 18%/CO 5%; ○, CO 5%; ▲, MO 5%/CO 18%. Life table data include all palpable tumors (adenocarcinoma and fibroadenoma). The proportion of animals surviving per unit time-without-tumor (1.0 represents 100% tumor-free animals) plotted as a function of days post-*N*-nitrosomethylurea (NMU) treatment. Tests for overall trend: Cox's test for adjusted trends and generalized Kruskal-Wallis analysis, not significant. Pairwise comparisons: Cox's test, Groups 3 and 4 *vs.* Groups 1, 2 and 5 ($P < 0.05$).

Groups 3 and 4 exhibited significant differences in tumor incidence, respectively.

Analysis of time-to-first-tumor curves for each of the five experimental groups (Fig. 3) indicated that mammary tumors appeared most rapidly in Group 3 (5% MO + 18% CO) and Group 4 (23% CO), less rapidly in Group 1 (15% MO + 18% CO) and least rapidly in Groups 2 (11.8% MO + 11.8% CO) and 5 (5% CO). Tests for overall trend were not significant, indicating that there was no direct (or indirect) relationship between the amount of MO in the diet and average time to first tumor in Groups 1–3. Pairwise comparisons by Wilcoxon's Rank test among the five experimental groups revealed that tumors appeared significantly more rapidly in Group 3 *vs.* Group 5 ($P < 0.001$), Group 2 *vs.* Group 3 ($P < 0.01$), and Group 2 *vs.* Group 4 ($P < 0.05$). Pairwise comparisons between Groups 1, 2 and 5 were nonsignificant. During the course of the experiment, palpable tumors occasionally appeared and then disappeared. The number of such animals in each group exhibiting such transient tumors were 4, 0, 4, 3, 2, respectively, in Groups 1–5.

The frequency distribution of tumors can be seen in Tables 4 and 5. Examination of the patterns of distribution of tumors/rat among the various groups reveals distinct differences. The three high incidence groups (1, 3 and 4) exhibited a pattern characterized by a higher frequency of animals bearing either one or two tumors than zero tumors. The two low-incidence groups (2 and 5) exhibited the opposite pattern. In terms of total tumor number, Groups 1, 3 and 4 exhibited significantly higher tumor yield compared to Groups 2 and 5 (Table 4). A similar pattern was found when only histologically verified adenocarcinomas (Table 5) were evaluated with the exception that total tumor yields in Group 4 were not significantly greater than in Group 2. When tumor multiplicity was assessed in terms of mean number of

TABLE 3

Mammary Tumor Incidence as a Function of Dietary Group

Group	Number rats with mammary tumor[a] *vs.* number rats at risk	%	Number rats with adenocarcinoma[b] *vs.* number rats at risk	%
1. Corn oil (5%) Menhaden oil (18%)	15/36	42	13/36	36
2. Corn oil (11.5%) Menhaden oil (11.5%)	16/36	44	11/36	30
3. Corn oil (18%) Menhaden oil (5%)	24/36	67	22/36	61
4. Corn oil (23%)	18/36	50	16/36	44
5. Corn oil (5%)	9/36	25	8/36	22

[a]All mammary tumors. Differences between Groups 3 and 4 *vs.* Group 5 are statistically significant ($P < 0.05$) based on the *Chi*-Square test; all other pairwise comparisons, NS.
[b]Adenocarcinomas only. Differences between Group 3 *vs.* 5 and Group 3 *vs.* 2 are statistically significant ($P < 0.05$) based on the *Chi*-Square test. All other pairwise comparisons, not significant.

TABLE 4

Frequency Distribution of Total Mammary Tumors by Experimental Group

Group	Number of tumors 0	1	2	3	4	7	Total tumors[a]	Mean number of tumors[c] per group
1. Corn oil (5%) Menhaden oil (18%)	21[b]	11	0	2	1	1	28	0.78 ± 1.46
2. Corn oil (11.5%) Menhaden oil (11.5%)	24	8	4	0	0	0	16	0.48 ± 0.70
3. Corn oil (18%) Menhaden oil (5%)	12	16	8	0	0	0	32	0.88 ± 0.76
4. Corn oil (23%)	18	12	5	0	1	0	26	0.83 ± 0.96
5. Corn oil (5%)	27	7	2	0	0	0	11	0.31 ± 0.57

[a]Pairwise comparisons. Differences between Group 2 *vs.* Groups 1, 3 or 4 and Group 5 *vs.* Groups 1, 3 or 4 are statistically significant ($P < 0.05$-0.01). All other pairwise comparisons, not significant.
[b]Number of rats.
[c]Mean ± SD. Group 3 *vs.* Group 2 ($P < 0.01$), Group 3 *vs.* Group 5 ($P < 0.004$). All other pairwise comparisons, not significant (analysis of variance).

TABLE 5

Frequency Distribution of Mammary Adenocarcinomas by Experimental Group

Group	Number of adenocarcinomas 0	1	2	3	4	5	Total tumors[a]	Mean number of tumors[c] per group
1. Corn oil (5%) Menhaden oil (18%)	21[b]	10	0	2	1	1	25	0.70 ± 1.2
2. Corn oil (11.5%) Menhaden oil (11.5%)	26	6	3	1	0	0	15	0.42 ± 0.77
3. Corn oil (18%) Menhaden oil (5%)	14	16	6	0	0	0	28	0.78 ± 0.72
4. Corn oil (23%)	19	12	4	0	1	0	24	0.67 ± 0.89
5. Corn oil (5%)	27	8	1	0	0	0	10	0.28 ± 0.51

[a]Pairwise comparisons. Differences between Group 3 *vs.* Group 5, Group 3 *vs.* Group 2, and Group 4 *vs.* Group 5 are statistically significant ($P < 0.05$-0.01) by *Chi* Square test.
[b]Number of rats.
[c]Mean ± SD. Group 3 *vs.* Group 5 ($P < 0.01$), Group 3 *vs.* Group 2 ($P < 0.0125$). All other pairwise comparisons, not significant (analysis of variance).

tumors/total animals at risk, or mean number of tumors/tumor bearing animal, no overall trends among the five treatment groups were observed. Rats in Group 3 exhibited higher multiplicities than Groups 2 and 5 ($P < 0.004$ and $P < 0.01$, respectively); all other pairwise comparisons were nonsignificant (Table 4). Similar results were obtained when only adenocarcinomas were compared. When assessed using only tumor bearing animals, Group 1, containing the highest levels of n-3 PUFA, exhibited greater tumor multiplicity than Groups 2-5 ($P < 0.001$) (Table 4).

Mean serum cholesterol levels (Table 6) were significantly suppressed in the high MO Groups (1 and 2) compared to the CO Groups (4 and 5). When assessed in terms of median levels, a similar pattern was observed (30, 36, 53, 58, 64 mg cholesterol/100 mL for Groups 1-5, respectively). Tests for trend indicated a strong inverse association between the proportion of MO in the diet and total serum cholesterol levels. No association was found between animal weight (at termination) or latency and serum cholesterol, nor was there any association between the presence or absence of a mammary tumor and serum cho-

TABLE 6

Serum Total Cholesterol as a Function of Diet[a]

Group	N[b]	Mean ± SD (median)	Range
1. Corn oil (5%) Menhaden oil (18%)	36	39 ± 13[c] (30)	30-78
2. Corn oil (11.5%) Menhaden oil (11.5%)	33	40 ± 13 (36)	30-77
3. Corn oil (18%) Menhaden oil (5%)	34	53 ± 19 (53)	30-88
4. Corn oil (23%)	29	58 ± 21	30-107
5. Corn oil (5%)	36	64 ± 15 (64)	30-97
Total	168	51 ± 19	30-107

[a]Statistical comparisons. Test for linear trend (Duncan's Multiple Range test), among Groups 1-4 ($P < 0.001$) based on multiple regression analysis adjusting for differences in weight and tumor latency. Pairwise comparisons. Group 1-4 *vs.* 5 ($P < 0.001$), Group 4 *vs.* 5 ($P < 0.002$), Group 1 *vs.* 5 ($P < 0.001$), Group 1 *vs.* Group 2 (not significant), Group 3 *vs.*. Group 4 (not significant) after adjustment for differences in weight and presence or absence of tumors by analysis of covariance.
[b]N = number of rats assayed.
[c]In mg/100 mL serum.

TABLE 7

Serum Fatty Acid Profiles

Group	Fatty acid						
	16:0	18:0	18:1n-9	18:2n-6	20:4n-6	20:5n-3	22:6n-3
1. Corn oil (5%) Menhaden (18%)	26(1.7)[a,b]	18(1.5)	12(3.5)	12(3.6)	5(1.6)	7(3.7)	2(0.7)
2. Corn oil (11.5%) Menhaden oil (11.5%)	26(2)	19(2)	11(1.5)	22(0.4)	7(0.6)	4(0.9)	1(0.5)
3. Corn oil (18%) Menhaden oil (5%)	24(6)	20(7)	13(1.6)	31(1)	11(1.5)	2(0.2)	1(0.2)
4. Corn oil (23%)	19(0)	16(2)	13(1)	31(3)	15(2.3)	0.2(0)	0.5(0.1)
5. Corn oil (5%)	25(0.6)	18(1)	14(0)	16(2.5)	15(1)	0.17(0.04)	0.4(0)

[a]Percent total fatty acids (SD). Rows do not necessarily add up to 100% since only the even major fatty acids in serum are shown. A total of 19 to 21 fatty acids were detected in groups 2, 3, 4 and 5. Group 1 exhibited 27 different fatty acids. In the majority of cases these minor fatty acids were present at <1% of the total.
[b]N = 3.

lesterol levels in individual animals. The results remained unchanged after adjustment for differences in weight gain and/or presence of a tumor or after segregation of cholesterol data into tertiles (30, 30–50, >50 mg/100 mL) followed by evaluation of the association between cholesterol and tumorigenesis by the Mantel-Haenszel *Chi* Square test and logistic regression.

Analysis of serum fatty acid profiles (Table 7) indicated that when dietary MO was increased at the expense of CO, keeping total lipid content constant, there was a stepwise decrement in the major n-6 serum fatty acids, linoleic (LA) and arachidonic (AA) and a concomitant stepwise increase in the major n-3 fatty acids, eicosapentaenoic (EPA) and docosohexaenoic (DHA). In contrast, lowering CO intake from 23 to 5% resulted in a 50% decrease in serum LA but no change in AA. No trend was found between the amount of EPA or DHA in serum and overall tumor yield in the five experimental groups. Interestingly, the levels of n-3 PUFA in serum never rose above 9% of total serum FA, despite levels of dietary n-3 intake as high as 4 g/100 g diet (Group 1).

DISCUSSION

In the present study, significant inhibition of mammary tumor promotion was seen only when MO and CO were fed in equal proportions (n-3/n-6 ratio = 0.55). Moreover, at the lowest MO/CO ratio (0.19) a nonsignificant enhancement of tumor numbers and incidence was seen. An HF diet containing a n-3/n-6 ratio of 0.55 resulted in a tumor yield (Tables 4 and 5) and latency (Fig. 3) similar to that of animals fed a LF CO diet indicating the importance of the type as well as the amount of dietary fat in affecting mammary tumor promotion.

Previous attempts at demonstrating linear dose-related effects of n-3 PUFA have been largely unsuccessful. Using a transplantable mouse mammary tumor model, Gabor and Abraham (16) examined the effects of various combinations of MO and CO fed at 10% (w/w) on tumor growth rates, and reported no dose-related changes when MO was increased from 25 to 75% of total fat. These authors did report, however, that growth of the tumor implant was inhibited at a MO/CO ratio of 9:1 or when MO alone was fed. In another study, using a transplantable rat mammary tumor model, Karmali *et al.* (8) fed F-344 rats (15/group) a 5% chow diet supplemented with Max EPA, a fish oil concentrate containing 17% EPA and 16% DHA (MO contains 16% EPA and 11% DHA and a far greater spectrum of other fatty acids), at 100, 200 and 400 μL rat/day. Growth of the tumor implant was inhibited equally in all three supplemented diets. Direct comparison of these studies with ours is difficult due to differences in (i) the resolving power of the models used, (ii) the small numbers of animals used in the transplant studies (5–15/group) and (iii) differences in the fat content and composition of the diets employed.

In the only study directly comparable to ours, Jurkowski and Cave (15) fed HF diets containing MO alone and MO/CO ratios of 3:1 and 1:3 to Buffalo rats initiated with NMU. No differences in tumor yields were found between the MO-containing groups and CO controls with the exception of the group fed MO alone which did suppress tumor development. Unfortunately, the authors did not include a group fed MO and CO in equal proportions. The results of the Jurkowski and Cave (15) study are consistent with our findings as are two other reports in the 7,12-dimethylbenz(a)anthracene (DMBA) model (10,17): low levels of MO in a CO-based diet either had no effect or enhanced mammary tumor development compared to CO alone. Also concordant with our findings is the study by Ip *et al.* (14) which reported that DMBA-tumor suppression occurred in rats fed almost equal levels of MO and CO (12% MO + 8% CO), but not in rats fed high levels of MO (19% MO + 1% CO). Interestingly, the tumor-enhancing effects of low levels of menhaden oil have also been observed in the neoplastic pancreatic foci model (26), the two-stage skin papilloma model (46) and the azoxymethanol-induced colon cancer model (23).

Various mechanisms have been proposed to explain the putative beneficial effects of n-3 PUFA. These include effects on eicosanoid metabolism (8,26,47–50), immune functions (50), cell membrane structure and function (50,51), free radical activity (9) and oncogene activity (47). Clearly, any mechanism proposed must be able to account or the nonlinear dose-response effect of n-3 PUFA in the mammary tumor model. In this regard, an eicosanoid-based mechanism seems plausible since eicosanoids have been shown to exhibit opposed biological effects based on their relative concentrations in specific tissues (52).

Most biochemical studies on n-3 PUFA have focused

on their effects on lipid metabolism and particularly serum triglyceride and cholesterol levels (39,53,54). In humans, n-3 PUFA suppress serum triglycerides and inconsistently suppress serum cholesterol (54), while in rodents both lipid classes are suppressed (39,53). Total serum cholesterol was examined in the present study because of the known link between high cholesterol and HF intake (55) and a reported epidemiological association between elevated serum cholesterol levels and (i) increased risk for breast cancer (56,57) and (ii) decreased survival among breast cancer patients (58,59). Our studies show clearly that serum cholesterol levels are inversely related to the amount of n-3 PUFA in the diet but are unrelated to mammary tumorigenesis.

Examination of serum FA profiles indicated that, in general, the serum profiles of the various treatment groups reflected the n-3/n-6 content of the diet, and because the levels of AA in the high MO groups were decreased, support the idea that increased n-3 PUFA suppresses elongation and desaturation of LA to AA. However, since we did not examine tissue PUFA profiles, it is not possible to know whether or not similar changes took place at the phospholipid or neutral lipid level. Two studies (8,15) have examined changes in mammary tumor membrane lipids induced by n-3 PUFA, and these suggest that n-3 PUFA can displace n-6 PUFA from membrane phospholipid and may thereby alter eicosanoid metabolism (8,18). It is of interest that despite the fact that the Group 1 diet provided twice as much n-3 PUFA as LA, serum levels of n-3 PUFA in Group 1 were lower (9%) than those of LA (12%). These results imply that dietary n-3 PUFA were preferentially oxidized or sequestered in a manner different than dietary LA.

It is noteworthy that the blended oils differed in a number of ways other than the relative amounts of n-3 and n-6 PUFA (Fig. 1). Fish oil, in contrast to corn oil, contains a wide variety of minor fatty acids, hence blends of CO and MO result in markedly different overall FA profiles. For example, 14:0 increased incrementally along with EFA and DHA, and the ratio of 18:0 to 18:2n-6 changed dramatically as the proportion of MO increased relative to CO. The possibility must therefore be considered that the effects of these diets on mammary tumorigenesis may not be due solely to n-3 PUFA or their n-3/n-6 ratios. To resolve this methodological problem future studies may require use of the nontriglyceride forms of n-3 PUFA as recently suggested by Ackman (60).

Several authors have drawn attention to the present dietary imbalance of n-3 and n-6 PUFA in Western countries and have attributed the high prevalence of chronic diseases such as coronary heart disease, cancer and arthritis in these societies specifically to this imbalance (66). With regard to breast cancer, this notion has received partial support from epidemiological studies (67-69). For example, special populations such as rural Japanese (70) and Greenland Eskomos (71), which exhibit low breast cancer rates, consume n-3/n-6 PUFA at ratios of 0.365 and >1, respectively, compared to high-risk Americans who consume a n-3/n-6 PUFA ratio of approximately 0.060 (71). Moreover, in a cross-country comparison, a positive association was demonstrated between breast cancer mortality and fish consumption (67), and Hislop *et al.* (68) reported similar results in a case/control study. In contrast, however, no association between risk for breast cancer and fish consumption was found in a recent large-scale prospective study (69).

In summary, the results of our study suggest that, with regard to the NMU-tumor inhibition, a n-3/n-6 ratio of 0.55 was the most effective and that raising or lowering the ratio above or below that point offered no beneficial effects. The implications of these experimental findings to human breast cancer remain to be determined.

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Effects of Dietary Proteins on Linoleic Acid Desaturation and Membrane Fluidity in Rat Liver Microsomes

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The effect of dietary protein, casein (CAS) and soybean protein (SOY), on linoleic acid desaturation in liver microsomes was studied in rats. The activity of Δ6 desaturase in total and rough endoplasmic reticula (ER and RER) was significantly higher in the CAS group than in the SOY group. In ER and smooth endoplasmic reticulum, the steady-state fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene, when incorporated into the membrane, was decreased in the SOY group and accompanied by a reduction in the cholesterol/phospholipid (CHOL/PL) ratio, consistent with an increase in membrane fluidity. In a separate study, the effect of varying dietary proteins, CAS, milk whey protein, egg albumin, SOY, potato protein and wheat gluten, on the relationship between the Δ6 desaturase activity and microsomal membrane fluidity was also examined. The results indicated that the dietary protein-dependent change in the liver microsomal CHOL/PL ratio affected membrane fluidity, and subsequently the activity of Δ6 desaturase in liver microsomes. However, since dietary protein influenced the Δ6 desaturase activity in RER without influencing membrane fluidity, it is possible that some regulation might have taken place at the level of enzyme synthesis.
Lipids 28, 457-464 (1993).

Dietary protein influences not only the serum cholesterol level (1,2) but also the metabolism of linoleic acid (3,4). It has been observed in rats that casein (CAS) not only increases the concentrations of serum and liver cholesterol, but also promotes the metabolism of linoleic acid to arachidonic acid more than does soybean protein (SOY) (3-8). These protein-dependent effects may result, at least in part, from the arginine content of the two dietary proteins (6,7). However, the exact mechanism as to how dietary protein affects linoleic acid metabolism is not clear. Since both Δ5 and Δ6 desaturases, two rate-limiting enzymes of linoleic acid metabolism, are bound to the microsomal membrane (9), it is possible that desaturase activity may be affected by changes in the liver microsomal membrane architecture. Microsomes (endoplasmic reticulum; ER) are composed of rough endoplasmic reticulum (RER), with ribosomal particles attached on the outer surface, and smooth endoplasmic reticulum (SER), which has no ribosomes attached (10). To elucidate the mechanism of the protein-dependent effect on the metabolism of linoleic acid, we invstigated the protein-effect at the level of RER and SER of rat liver. We also studied the relationship between membrane fluidity and Δ6 desaturase activity and between membrane fluidity and the relative distribution of liver microsomal lipids [cholesterol/phospholipid (CHOL/PL) ratio and phospholipid subclasses] using various dietary proteins.

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Abbreviations: CAS, casein; CHOL, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; EAL egg albumin; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; POT, potato protein; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; SOY, soybean protein; TLC, thin-layer chromatography; WGL, wheat gluten; WHY, milk whey protein.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats (four-weeks-old) were purchased from Seiwa Experimental Animals (Fukuoka, Japan) and acclimated for four days (Experiment 1) or three days (Experiment 2) in a room maintained at 20-23°C with a 12-h light-dark cycle (lights on 8 a.m. to 8 p.m.). During this period, rats were allowed free access to commercial rat chow (Type NMF, Oriental Yeast Co., Tokyo, Japan). Then rats were divided into two groups (Experiment 1) and six groups (Experiment 2) of six animals each according to the source of the dietary protein. In Experiment 1, the dietary proteins used were CAS (Wako Pure Chemical Industries Ltd., Osaka, Japan) and SOY (Fujipro R, Fuji Oil Co., Osaka, Japan). In Experiment 2, CAS, milk whey protein (WHY; Meiji Milk Co., Tokyo, Japan), egg albumin (EAL; Wako Pure Chemical Industries Ltd.), SOY, potato protein (POT; protamyl PF; Avebe Holland, provided by Ajinomoto Co., Tokyo, Japan) and wheat gluten (WGL; Nisshin Flour Milling Co., Tokyo, Japan) were used. The diets were prepared according to recommendations of the American Institute of Nutrition (11) and contained (by wt%) protein (CAS or SOY) 20, corn oil (Ajinomoto Co.) 5.0, vitamin mixture (AIN-76™) 1.0, mineral mixture (AIN-76™) 3.5, choline bitartrate 0.2, DL-methionine 0.3, cellulose 5.0, corn starch 15 and sucrose 50 for Experiment 1. In Experiment 2, the dietary protein level (wt%) was adjusted to be isonitrogenous as follows: CAS 20, WHY 20, EAL 21.8, SOY 20, POT 20, WGL 25.7 + 0.7% L-lysine·HCl + 0.32% $NaHCO_3$. The diets were adjusted to 100% at the expense of corn starch. In both experiments, the rats were fed these diets *ad libitum* for 21 d. At the end of the feeding period, they were killed by decapitation, and liver was excised immediately.

Preparation of liver microsomes (total ER fraction). According to the method of Svensson (12), a piece of liver (approximately 2 g) was homogenized in 10 vol of 0.25 M sucrose solution containing 0.05 M potassium phosphate buffer (pH 7.0), 0.15 M KCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1.5 mM reduced glutathione. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was recentrifuged at 105,000 × *g* for 60 min to sediment microsomes. The microsomal pellet was resuspended in potassium phosphate buffer (pH 7.0) (approximately 10 mg protein/mL).

Separation of liver RER and SER fractions. According to the method of Dallner (13), a piece of liver (approximately 2 g) was homogenized in 12 mL of 0.25 M sucrose buffer containing 0.05 M potassium phosphate buffer (pH 7.0), 0.15 M KCl, 5 mM $MgCl_2$, 1 mM EDTA and 1.5 mM

reduced glutathione. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was harvested. Then 0.15 mL of 1 M CsCl was added to 9.85 mL of this supernatant giving a final CsCl concentration of 15 mM. The supernatant (7 mL) was layered over 4.5 mL of the buffer containing 1.30 M sucrose and 15 mM CsCl (pH 7.0). After centrifugation at 165,000 × *g* for 180 min at 4°C, a fluffy double-layered fraction appearing around the gradient boundary and a tight pellet at the bottom (RER fraction) were separated. The upper 7.5 mL including the fluffy double-layer was diluted with 4 mL 0.25 M sucrose buffer (pH 7.0) and recentrifuged at 165,000 × *g* for 60 min at 4°C to yield a tight pellet (SER fraction). Each pellet was resuspended with 0.25 M sucrose buffer (pH 7.0). We confirmed that CsCl that may be contaminating the RER fraction does not influence the enzymatic activity.

Measurement of the desaturase activity of liver microsomes. [1-^{14}C]Linoleic acid (51.7 mCi/mmol, New England Nuclear, Boston, MA) and [1-^{14}C]dihomo-γ-linolenic acid (47.0 mCi/mmol, New England Nuclear) were used as substrates to determine the activities of the Δ6 and Δ5 desaturase, respectively. Enzyme activity measurements were based on the method of Svensson (12). Microsomal protein (approximately 1 mg) was incubated at 37°C for 20 min together with 100 nmol 0.1 μCi radiolabelled fatty acid in 1 mL (final volume) 0.25 M sucrose buffer containing 0.1 M KCl, 5 mM ATP, 0.25 mM CoA, 0.25 mM NADH, 5 mM $MgCl_2$, 1.5 mM reduced glutathione, 45 mM NaF and 0.5 mM nicotinamide. In a preliminary study, we found that under these conditions the enzymes were saturated with substrate and the reactions proceeded linearly with microsomal protein concentration and incubation time (12). The reaction was terminated by adding 5 mL of ethanol, and lipids were extracted. Fatty acids were separated by argentation thin-layer chromatography (TLC) (14) using petroleum ether/diethyl ether (70:30 and 30:70, vol/vol, for Δ6 and Δ5 desaturase, respectively) as a developing solvent. The fractions corresponding to fatty acids with 2 and 3 double bonds were scraped for the measurement of Δ6 desaturase activity, whereas those with 3 and 4 double bonds were scraped for the measurement of Δ5 desaturase activity. Each fraction dissolved in a toluene scintillation fluid (2 g of 2,5-diphenyloxazole and 50 mg of 2,2′-*p*-phenylene-*bis*[5-phenyloxazole]/liter toluene), and radioactivity was determined using a liquid scintillation spectrometer (Aloka LSC-1000, Aloka, Tokyo, Japan). Microsomal protein was measured by the method of Lowry *et al.* (15).

Lipid analyses. Liver microsomal lipids were extracted by the method of Folch *et al.* (16). CHOL was assayed by gas-liquid chromatography (GLC) on a OV-17 column (17). Microsomal PLs were resolved by TLC (18). The concentration of total PL as well as of individual PL classes was measured using the method of Rouser *et al.* (19). Fatty acid compositions were analyzed by GLC of the methyl esters using a SILAR 10C (Chromatotec, Tokyo, Japan) column (20).

Fluorescence anisotropy measurements. According to the method of Leikin and Brenner (21), one volume of 2 μM 1,6-diphenyl-1,3,5-hexatriene (DPH; Nacalai Tesque, Kyoto, Japan) was mixed with a volume of a microsomal suspension diluted with 0.25 M sucrose buffer (pH 7.0) (approximately 50 μM phospholipid). Then the mixture was incubated for 1 h at 25°C. A reference blank was prepared without the fluorescence probe. The fluorescence anisotropy measurements (352 nm excitation and 435 nm emission) were made at 37°C in a spectrofluorometer equipped with two polarizers (Hitachi 650-10S, Tokyo, Japan) with a HP-85F computer (Yokogawa Hewlet-Packard, Tokyo, Japan). They were corrected by subtraction of the corresponding blank values. The steady-state fluorescence anisotropy (r_s) was calculated using the equation: $r_s = (I_\parallel - GI_\perp)/(I_\parallel + 2GI_\perp)$ where $I_\parallel$ and $I_\perp$ are the fluorescence intensities detected with the excitation polarizer in vertical position and with the analyzer in vertical and horizontal position, respectively. $G = i_\perp/i_\parallel$ is a correction factor arising from instrumental factors. The fluorescence intensities are $i_\perp$ and $i_\parallel$ detected with the excitation polarizer in horizontal position and the analyzer in vertical and horizontal position, respectively.

Statistical analyses. Data were analyzed by Student's *t*-test (22) or Duncan's new multiple-range test (23) preceded by analysis of variance.

RESULTS

Growth and liver weight. In Experiment 1, no statistically significant differences were found in body weight gain and food consumption between the groups (Table 1). Relative liver weight (g/100 g body weight) was higher in rats fed the CAS diet than in those fed the SOY diet. In Experiment 2, body weight gain was lower in the EAL group than in the SOY group, although food consumption was comparable among the groups (Table 1). Relative liver weight was significantly higher in rats fed the CAS diet than in those fed the EAL, SOY, POT and WGL diets and was lowest in the SOY group. When the effects of animal proteins were compared to those of vegetable protein, relative liver weight was significantly higher in the former than in the latter (5.80 ± 0.12 *vs.* 5.20 ± 0.07, $P < 0.05$).

Desaturase activity in liver microsomes. In Experiment 1, the Δ6 desaturase activity in total ER and RER fractions was significantly higher in the CAS group than in the SOY group (Fig. 1). The Δ5 desaturase activity of total ER and SER was also higher in the CAS group than in the SOY group, but differences were not statistically significant. The Δ5 desaturase activity of RER was comparable between the groups. In Experiment 2, the activity of Δ6 desaturase was significantly higher in rats fed CAS, EAL or POT diets than in those fed the SOY diet (Fig. 2). The activities of the WHY and WGL groups also tended to be lower than that of the CAS, EAL and POT groups, although the difference was not statistically significant. However, no effect was found in the activity between animal and vegetable proteins.

The activity of Δ6 desaturase measured in Experiments 1 and 2 was different (231 *vs.* 80 nmol/min·mg protein for CAS and 112 *vs.* 56 nmol/min·mg protein for SOY). In Experiment 1, the activity of total endoplasmic reticulum (microsomes) was measured after storage for about 6 h at 4°C until RER and SER were available. In Experiment 2, on the other hand, the enzymatic activity was measured immediately after separation of microsomes. These differences may influence the activity of desaturase. Nevertheless a dietary protein-dependent effect on the desaturase activity was observed in both experiments.

TABLE 1

Effects of Dietary Protein on Growth Parameters and Liver Weight[a]

Group	Body weight (g)		Food intake (g/d)	Liver weight (g/100 g body wt)
	Initial	Gain		
Experiment 1				
CAS	114 ± 2	185 ± 9	21.7 ± 0.8	6.18 ± 0.14[b]
SOY	114 ± 2	178 ± 10	20.7 ± 0.7	5.08 ± 0.05[b]
Experiment 2				
CAS	108 ± 4	179 ± 5[b,c]	21.5 ± 0.4	6.16 ± 0.22[b]
WHY	108 ± 3	169 ± 7[b,c]	20.4 ± 0.8	5.74 ± 0.22[b,e]
EAL	108 ± 3	160 ± 11[b]	19.4 ± 0.9	5.51 ± 0.14[c,e]
SOY	108 ± 3	188 ± 8[c]	21.5 ± 0.7	4.91 ± 0.05[d]
POT	108 ± 3	175 ± 11[b,c]	20.7 ± 1.0	5.25 ± 0.06[c,d]
WGL	108 ± 3	167 ± 8[b,c]	20.0 ± 0.8	5.45 ± 0.10[c,e]

[a]Mean ± SE of six rats. CAS, casein; SOY, soybean protein; WHY, milk whey protein; EAL, egg albumin; POT, potato protein; WGL, wheat gluten. Values in each experiment without a common superscript (b-e) are significantly different at $P < 0.05$.

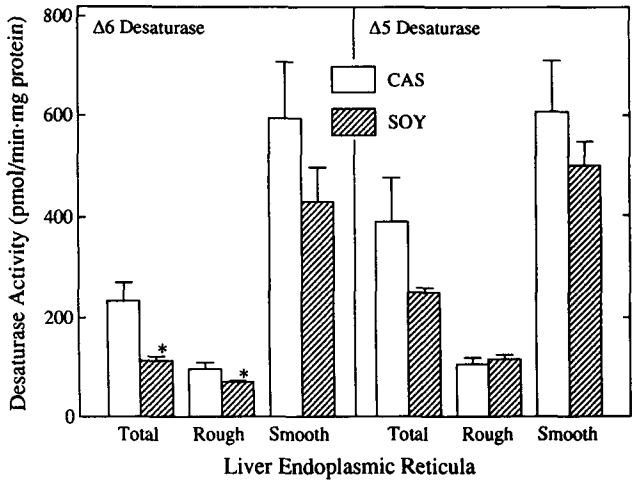


FIG. 1. Effects of dietary protein on Δ6 and Δ5 desaturase activities of liver endoplasmic reticula (Experiment 1). Values are expressed as mean ± SE of six rats. CAS, casein; SOY, soybean protein. *Significantly different from the corresponding CAS group at $P < 0.05$.

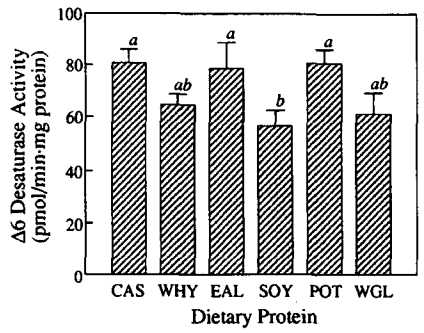


FIG. 2. Effects of dietary protein on Δ6 desaturase activity of liver microsomes (Experiment 2). Values are expressed as mean ± SE of six rats. CAS, casein; WHY, milk whey protein; EAL, egg albumin; SOY, soybean protein; POT, potato protein; WGL, wheat gluten. *a,b*Values without a common superscript are significantly different at $P < 0.05$.

Lipid composition of liver microsomes. The lipid composition of hepatic ER (Experiment 1) is shown in Table 2. The concentration of CHOL in SER was significantly lower in the SOY group than in the CAS group. RER contained approximately one-fifth to one-sixth as much CHOL as SER, but no protein-dependent difference was observed. Similarly, the PL concentration in ER was approximately one-third of that seen in SER. No protein effect on the PL concentration was observed in all fractions. The CHOL/PL ratio of total ER and SER was significantly higher in the CAS group than in the SOY group, whereas the CHOL/PL ratios of the RER were comparable between the groups. In Experiment 2, the concentration of CHOL was significantly higher in the CAS, EAL and POT groups than in the WHY, SOY and WGL groups (Table 2). The PL concentration was significantly higher in the CAS, EAL and POT groups than in the WGL group, whereas the PL levels in the WHY and SOY groups were comparable. As a result, the CHOL/PL ratio of the CAS group was significantly higher than that of the SOY and WGL groups. When the effects of animal proteins were compared to those of vegetable proteins, the concentration of CHOL (39.4 ± 0.6 *vs.* 37.2 ± 0.7, $P < 0.05$) and the CHOL/PL ratio (9.22 ± 0.11 *vs.* 8.81 ± 10, $P < 0.05$) were significantly higher in the former than in the latter. Although it is difficult to explain the reason why the lipid concentrations observed in Experiment 1 were higher than those in Experiment 2, the ratio of CHOL/PL was comparable, and the same protein-dependent difference was observed in both experiments.

Fatty acid composition of liver microsomal lipids. The fatty acid composition of total lipids in hepatic ER (Experiment 1) is shown in Table 3. The proportion of linoleic acid was significantly lower in the CAS group than in the SOY group in all ER fractions. The proportion of arachidonic acid in total ER was significantly higher in the CAS group than in the SOY group, whereas those in RER and SER were comparable between the groups. Consequently, the desaturation index for linoleic acid expressed as (20:3n-6 + 20:4n-6)/18:2n-6 was significantly higher in the CAS group than in the SOY group in all ER fractions. Interestingly, the RER fraction in both dietary protein groups, contained more n-3 highly polyunsaturated fatty

TABLE 2

Effects of Dietary Protein on Lipid Composition of Hepatic Endoplasmic Reticulum (Microsomes)[a]

Group	Lipid (nmol/mg protein)		CHOL/PL ($\times 10^2$)
	CHOL	PL	
Experiment 1			
Total endoplasmic reticulum			
CAS	145 ± 21	1147 ± 203	12.9 ± 1.1[b]
SOY	95.5 ± 7.8	969 ± 73	9.99 ± 0.36[c]
Rough endoplasmic reticulum			
CAS	27.8 ± 1.5	340 ± 30	8.31 ± 0.39
SOY	30.2 ± 2.4	359 ± 19	8.43 ± 0.44
Smooth endoplasmic reticulum			
CAS	180 ± 6[b]	1187 ± 23	15.2 ± 0.6[b]
SOY	162 ± 4[c]	1196 ± 17	13.5 ± 0.4[c]
Experiment 2			
Liver microsomes			
CAS	40.8 ± 0.7[b]	435 ± 10[b]	9.38 ± 0.16[b]
WHY	37.3 ± 1.1[c,d]	413 ± 7[b,c]	9.03 ± 0.19[b,c]
EAL	40.1 ± 0.8[b,d]	434 ± 5[b]	9.24 ± 0.24[b,c]
SOY	36.5 ± 1.1[c,e]	418 ± 7[b,c]	8.73 ± 0.21[c]
POT	39.2 ± 1.2[b,d,e]	436 ± 11[b]	8.99 ± 0.08[b,c]
WGL	35.4 ± 1.0[c]	407 ± 6[c]	8.69 ± 0.20[c]

[a]Mean ± SE of five or six rats. CHOL, cholesterol; PL, phospholipid; CAS, casein; SOY, soybean protein; WHY, milk whey protein; EAL, egg albumin; POT, potato protein; WGL, wheat gluten. Values in each experiment without a common superscript (b-e) are significantly different at $P < 0.05$.

acids, in particular docosapentaenoic acid (22:5n-3) than did the SER fraction.

In Experiment 2, the proportion of linoleic acid in liver microsomal phosphatidylcholine (PC) was significantly lower in rats fed the CAS, EAL and POT diets than in those fed the SOY and WGL diets (Table 3). Conversely, the proportion of arachidonic acid was significantly higher in rats fed the WHY, EAL and POT diets than in those fed the SOY diet. Consequently, the desaturation index, (20:3n-6 + 20:4n-6)/18:2n-6, was significantly higher in the CAS, WHY, EAL and POT groups than in the SOY and WGL groups. In rats fed WGL, all responses resembled those in rats fed SOY. When comparisons were made between animal and vegetable proteins, the proportion of linoleic acid (8.0 ± 0.2 *vs.* 9.0 ± 0.3, $P < 0.05$) was significantly lower whereas that of arachidonic acid (31.1 ± 0.3 *vs.* 29.9 ± 0.3, $P < 0.05$) was significantly higher in the former than in the latter. Consequently, the desaturation index was significantly higher in rats fed animal proteins than in those fed vegetable proteins (4.2 ± 0.1 *vs.* 3.7 ± 0.2, $P < 0.05$).

Distribution of PL subclasses in liver microsomes. The PL compositions are shown in Table 4. In Experiment 1, the protein-effect was observed only in the proportion of lysophosphatidylcholine and phosphatidylinositol, and no effect was seen in the main components, PC and phosphatidylethanolamine (PE) in all fractions except PC in SER. The ratio of PC/PE was therefore similar in all fractions.

In Experiment 2, the proportion of liver microsomal PC was significantly higher ($P < 0.05$) in the animal proteins (57.2 ± 0.5%) than in the vegetable protein group (55.4 ± 0.5%), whereas the proportion of PE was significantly lower ($P < 0.05$) in the animal protein groups (9.6 ± 0.2%) than in the vegetable protein group (10.7 ± 0.3%). Thus, the PC/PE ratio was significantly higher ($P < 0.05$) for animal protein fed rats (6.0 ± 0.2%) than for vegetable protein fed rats (5.3 ± 0.2%). The difference was most evident in the CAS and WHY groups in comparison with the WGL group (Table 4). The proportion of PC was significantly higher (57.2 ± 0.5 *vs.* 55.4 ± 0.5, $P < 0.05$) whereas that of PE was significantly lower (9.6 ± 0.2 *vs.* 10.7 ± 0.3, $P < 0.05$) in rats fed animal proteins than in those fed vegetable proteins.

Membrane fluidity measured by fluorescence anisotropy. In Experiment 1, the DPH-fluorescence anisotropy in total ER and SER was significantly higher in the CAS group than in the SOY group, but that in RER was comparable between the groups (Fig. 3). In Experiment 2, the fluorescence anisotropy of the CAS group was significantly higher than that of the SOY and WGL groups (Fig. 4). When the effect of animal proteins was compared to vegetable proteins, the fluorescence anisotropy was significantly higher in the former than in the latter (0.099 ± 0.003 *vs.* 0.089 ± 0.002, $P < 0.05$).

Correlations between lipid parameters, Δ6 desaturase activity and membrane fluidity. As shown in Figure 5, the fluorescence anisotropy was highly correlated with the CHOL/PL ratio (r = +0.941) (Fig. 5A). Significant correlation was also observed between the CHOL/PL ratio *vs.* Δ6 desaturase activity (r = +0.832) (Fig. 5B). The anisotropy *vs.* Δ6 desaturase activity (r = +0.804) was not correlated significantly ($0.05 < P < 0.1$) (Fig. 5C). The correlations between the anisotropy *vs.* the PC/PE ratio (r = +0.800) (Fig. 6A) and PC/PE ratio *vs.* the Δ6 desaturase activity (r = +0.420) were also not significant (Fig. 6B).

DISCUSSION

The present study showed a characteristic effect of dietary protein on the metabolism of linoleic acid. The results in

TABLE 3

Effects of Dietary Protein on the Polyunsaturated Fatty Acid Composition of Total Lipid of Hepatic Endoplasmic Reticula and of Phosphatidylcholine of Liver Microsomes[a]

Group	Fatty acid (wt%)						Desaturation index[b]
	18:2n-6	20:3n-6	20:4n-6	22:5n-6	22:5n-3	22:6n-3	
Experiment 1: total lipids							
Total endoplasmic reticulum							
CAS	6.3 ± 0.3^{c}	1.1 ± 0.2	19.0 ± 0.5^{c}	2.2 ± 0.1^{c}	2.2 ± 0.2	1.9 ± 0.1	3.3 ± 0.3^{c}
SOY	7.7 ± 0.3^{d}	1.0 ± 0.1	17.5 ± 0.5^{d}	1.9 ± 0.1^{d}	1.7 ± 0.2	2.1 ± 0.1	2.5 ± 0.1^{d}
Rough endoplasmic reticulum							
CAS	5.1 ± 0.2^{c}	0.8 ± 0.1	16.0 ± 0.7	2.4 ± 0.1	10.0 ± 1.1	3.8 ± 0.4	3.4 ± 0.1^{c}
SOY	6.1 ± 0.3^{d}	0.7 ± 0.2	16.5 ± 0.7	2.3 ± 0.3	9.1 ± 0.9	3.5 ± 0.4	2.9 ± 0.1^{d}
Smooth endoplasmic reticulum							
CAS	6.3 ± 0.3^{c}	1.0 ± 0.1^{c}	19.9 ± 0.4	2.8 ± 0.1	1.6 ± 0.2	2.1 ± 0.1^{c}	3.4 ± 0.2^{c}
SOY	7.8 ± 0.2^{d}	1.3 ± 0.1^{d}	19.0 ± 0.3	2.7 ± 0.2	1.5 ± 0.1	2.5 ± 0.1^{d}	2.7 ± 0.1^{d}
Experiment 2: phosphatidylcholine of liver microsomes							
CAS	7.5 ± 0.2^{c}	1.7 ± 0.0^{c,d}	30.5 ± 0.2^{c,e}	3.9 ± 0.3	—	2.4 ± 0.1^{c}	4.4 ± 0.1^{c}
WHY	8.5 ± 0.3^{c,e}	1.5 ± 0.1^{c}	31.5 ± 0.8^{c}	3.3 ± 0.2	—	2.2 ± 0.2^{c}	4.0 ± 0.2^{c}
EAL	8.0 ± 0.4^{c}	1.6 ± 0.2^{c}	31.3 ± 0.5^{c}	3.4 ± 0.2	—	3.0 ± 0.1^{d}	4.2 ± 0.2^{c}
SOY	9.8 ± 0.4^{d}	2.1 ± 0.1^{d}	28.6 ± 0.2^{d}	3.3 ± 0.4	—	2.9 ± 0.1^{d}	3.2 ± 0.1^{d}
POT	7.7 ± 0.6^{c}	1.8 ± 0.1^{c,d}	31.3 ± 0.6^{c}	4.0 ± 0.3	—	4.0 ± 0.2^{e}	4.5 ± 0.3^{c}
WGL	9.5 ± 0.4^{d,e}	1.7 ± 0.2^{c,d}	29.8 ± 0.4^{d,e}	3.4 ± 0.2	—	3.2 ± 0.2^{d}	3.4 ± 0.2^{d}

[a]Mean ± SE of six rats. CAS, casein; SOY, soybean protein; WHY, milk whey protein; EAL, egg albumin; POT, potato protein; WGL, wheat gluten. Values in each experiment without a common superscript (c-e) are significantly different at $P < 0.05$.

[b](20:3n-6 + 20:4n-6)/18:2n-6.

TABLE 4

Effects of Dietary Protein on Phospholipid Composition of Hepatic Endoplasmic Reticula[a]

Group	Phospholipid (mol%)							
	SPH	LPC	PC	PE	PI	PA	PS	PC/PE
Experiment 1								
Total endoplasmic reticulum								
CAS	3.1 ± 0.1	3.7 ± 0.2^{b}	62.5 ± 0.5	12.4 ± 0.4	10.3 ± 0.2^{b}	2.7 ± 0.2	3.2 ± 0.1	5.0 ± 0.2
SOY	3.2 ± 0.3	2.8 ± 0.1^{c}	61.6 ± 0.5	13.3 ± 0.3	11.7 ± 0.1^{c}	2.0 ± 0.1	3.1 ± 0.0	4.6 ± 0.1
Rough endoplasmic reticulum								
CAS	4.6	5.4	58.2	7.5	10.3	2.8	4.1	7.7
SOY	4.1	4.9	60.0	7.4	11.1	2.1	3.4	8.1
Smooth endoplasmic reticulum								
CAS	7.1 ± 0.3	7.3 ± 0.1	52.2 ± 0.5^{b}	8.5 ± 0.3	7.0 ± 0.2	2.2 ± 0.1	4.3 ± 0.2	6.1 ± 0.2
SOY	7.8 ± 0.3	7.4 ± 0.3	50.2 ± 0.7^{c}	8.6 ± 0.2	7.3 ± 0.2	2.1 ± 0.1	4.1 ± 0.2	5.9 ± 0.1
Experiment 2								
CAS	6.0 ± 0.4	5.4 ± 0.3	56.0 ± 1.2^{b,c}	9.1 ± 0.3^{b}	9.7 ± 0.4	2.8 ± 0.4^{b}	4.1 ± 0.2	6.2 ± 0.3^{b}
WHY	5.9 ± 0.2	4.7 ± 0.3	58.2 ± 0.9^{b}	9.5 ± 0.4^{b}	9.4 ± 0.3	2.6 ± 0.3^{b,d}	4.0 ± 0.2	6.2 ± 0.3^{b}
EAL	5.3 ± 0.6	4.9 ± 0.3	57.4 ± 0.3^{b,c}	10.2 ± 0.4^{b,c}	10.3 ± 0.6	2.3 ± 0.3^{b,c}	3.9 ± 0.1	5.7 ± 0.2^{b,c}
SOY	5.5 ± 0.2	5.3 ± 0.4	55.5 ± 0.8^{b,c}	10.4 ± 0.3^{b,c}	11.0 ± 0.8	1.9 ± 0.3^{c,d}	4.1 ± 0.2	5.4 ± 0.2^{b,c}
POT	5.8 ± 0.5	4.8 ± 0.3	55.4 ± 0.9^{b,c}	10.3 ± 0.6^{b,c}	10.4 ± 0.6	2.0 ± 0.2^{c,d}	3.6 ± 0.2	5.5 ± 0.4^{b,c}
WGL	6.0 ± 0.4	4.9 ± 0.4	55.1 ± 1.2^{c}	11.4 ± 0.7^{c}	10.8 ± 0.7	1.7 ± 0.1^{c}	3.7 ± 0.3	4.9 ± 0.4^{c}

[a]Mean ± SE of six rats, except for pooled values from six samples for rough endoplasmic reticulum. SPH, sphingolipids; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; CAS, casein; SOY, soybean protein; WHY, milk whey protein; EAL, egg albumin; POT, potato protein; WGL, wheat gluten. Values in each experiment without a common superscript (b-d) are significantly different at $P < 0.05$.

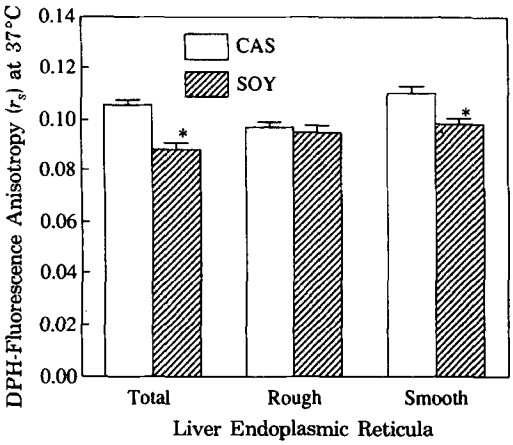


FIG. 3. Effects of dietary protein on fluorescence anisotropy of liver endoplasmic reticula (Experiment 1). Values are expressed as mean ± SE of six rats. CAS, casein; DPH, 1,6-diphenyl-1,3,5-hexatriene; SOY, soybean protein. *Significantly different from the corresponding CAS group at $P < 0.05$.

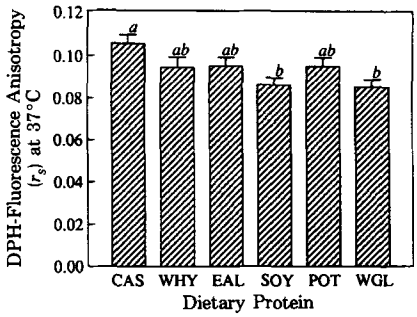
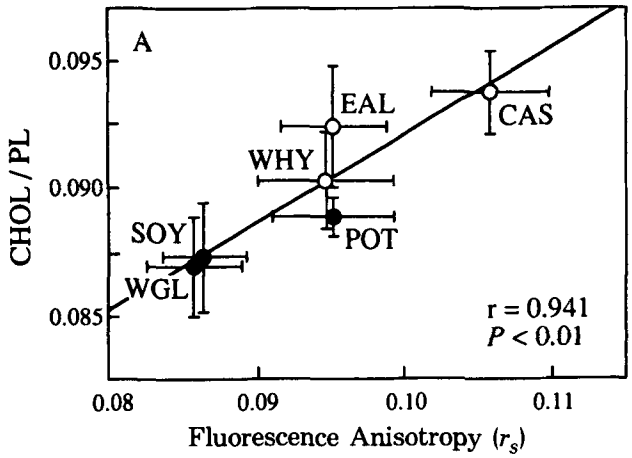
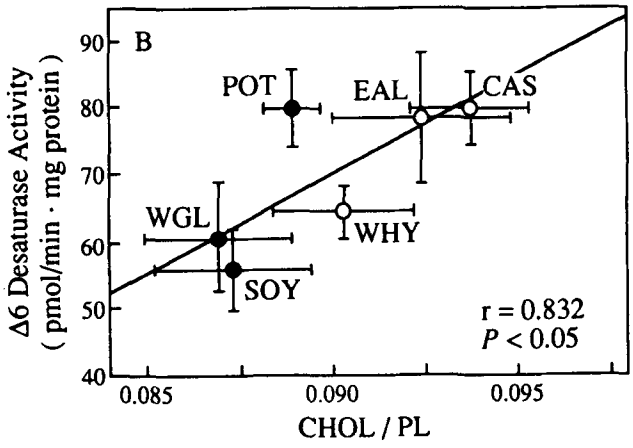


FIG. 4. Effects of dietary protein on fluorescence anisotropy of liver microsomes (Experiment 2). Values are expressed as mean ± SE of six rats. CAS, casein; WHY, milk whey protein; EAL, egg albumin; SOY, soybean protein; POT, potato protein; WGL, wheat gluten; DPH, 1,6-diphenyl-1,3,5-hexatriene. ^{a,b}Values without a common superscript are significantly different at $P < 0.05$.





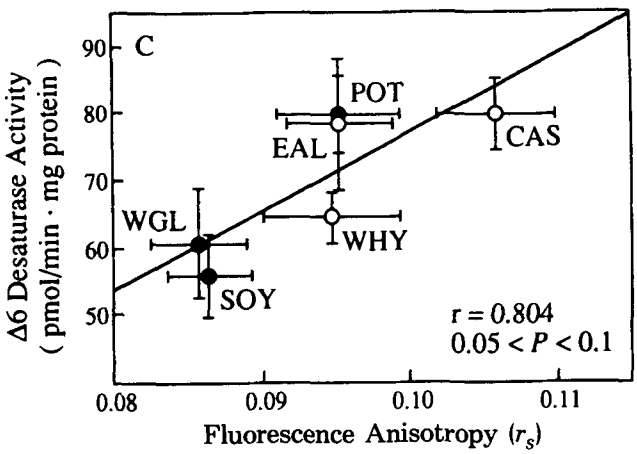


FIG. 5. Correlation between Δ6 desaturase activity, fluorescence anisotropy and microsomal lipids (Experiment 2). Values are expressed as mean ± SE of six rats. CAS, casein; WHY, milk whey protein; EAL, egg albumin; SOY, soybean protein; POT, potato protein; WGL, wheat gluten. CHOL/PL, cholesterol/phospholipid ratio; PC/PE, phosphatidylcholine/phosphatidylethanolamine ratio.

Experiment 1 showed that the Δ6 desaturase activity in total ER and RER in the CAS group was higher than that in the SOY group. This protein-dependent difference was also reflected in the fatty acid profiles of total PL in microsomal preparations; the Δ6 desaturation index expressed as (20:3n-6 + 20:4n-6)/18:2n-6 was significantly higher in ER, RER and SER in rats fed the CAS diet than in those fed the SOY diet. The results in Experiment 2 with various dietary proteins also indicated that microsomal membrane fluidity plays an important role in the regulation of the Δ6 desaturase activity of liver microsomes. Since the Δ5 desaturase activity in total ER and SER was also higher in the CAS group than in the SOY group, it is possible that dietary protein exerted a similar mode of the action on both desaturase activities, even though the response seemed to be less marked with Δ5 than with Δ6 desaturase.

The exact mechanism as to how dietary protein affects microsomal membrane properties is not clear. Previously, we (5–8) and others (24) have reported that CAS-feeding as compared with SOY-feeding stimulated the Δ6 desaturase activity of liver microsomes. Garda and Brenner (25) have shown in an *in vitro* study that a decrease in the fluoescence anisotropy (increased membrane fluidity) caused a decrease in the activity of Δ6 desaturase and that an increase in the fluorescence anisotropy (decreased membrane fluidity) induced an increase in Δ6 desaturase

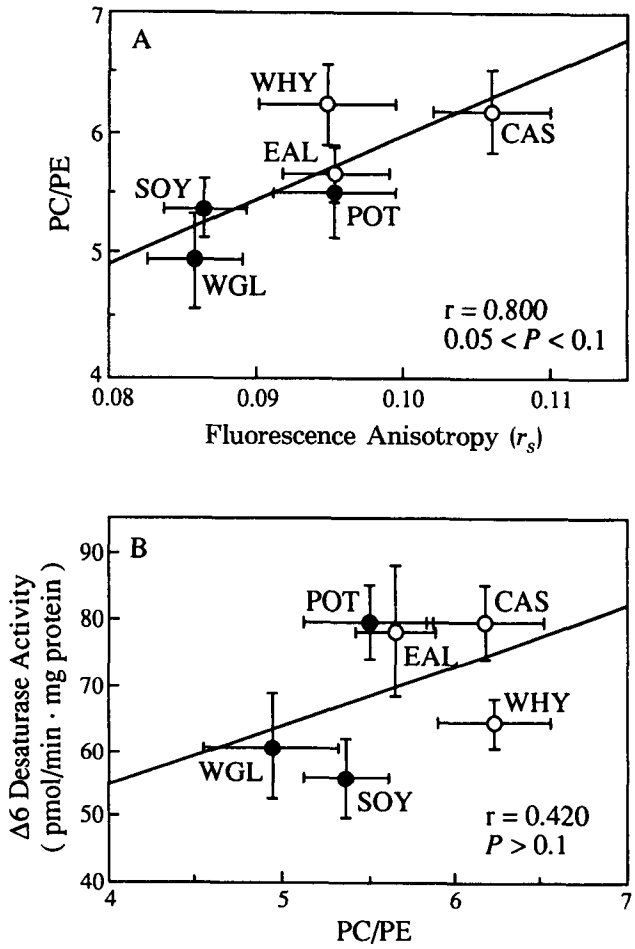


FIG. 6. Correlation between Δ6 desaturase activity, fluorescence anisotropy and microsomal lipids (Experiment 2). Values are expressed as mean ± SE of six rats. CAS, casein; WHY, milk whey protein; EAL, egg albumin; SOY, soybean protein; POT, potato protein; WGL, wheat gluten. CHOL/PL, cholesterol/phospholipid ratio; PC/PE, phosphatidylcholine/phosphatidylethanolamine ratio.

activity in liver microsomes (26). These findings suggest that the activity of desaturases is closely associated with membrane fluidity. Since the steady-state fluorescence anisotropy in the total ER was significantly higher in the CAS group than in the SOY group (Fig. 3), membrane fluidity was lower in the CAS than in the SOY groups. Furthermore, fluorescence anisotropy was correlated significantly with Δ6 desaturase activity (r = 0.804), indicating that an increase in the fluorescence anisotropy (decreased membrane fluidity) is associated with an increase in the total Δ6 desaturase activity (Fig. 5). Thus, our finding is consistent with the *in vitro* studies by Garda and Brenner (25,26). It is concluded that dietary protein modulates desaturase activity through its effect on membrane fluidity.

To examine how dietary protein affects membrane fluidity, we have also analyzed the microsomal lipid profiles. Results in Table 2 show that dietary protein modulated the lipid composition of liver ER; the ratio of CHOL/PL of total ER was significantly higher in the CAS group than in the SOY group. The evidence that the CHOL/PL ratio directly influences the microsomal membrane fluidity is further illustrated by the results shown in Figure 5, which illustrate that fluorescence anisotropy correlated significantly with the CHOL/PL ratio. Therefore, it is confirmed that dietary protein changes the microsomal CHOL/PL ratio, which in turn modulates membrane fluidity. Hence, Δ6 desaturase activity reflects changes in the fluidity of the microsomal membrane consistent with the "self-regulatory function" of liver microsomes proposed by Garda and Brenner (25,26). Since these protein-dependent differences in the CHOL/PL ratio and membrane fluidity were detected only in SER, but not in RER, it seems that the above mechanism operates only in SER. However, the results have shown that dietary protein also modulated Δ6 desaturase activity in RER, even though the CHOL/PL ratio and the membrane fluidity of RER were not affected. Since the microsomal desaturase enzyme is considered to be synthesized on the RER (27), it is speculated that CAS, in comparison with SOY, might have stimulated the synthesis of the Δ6 desaturase enzyme protein, which then exhibited its activity after being transferred to the SER.

The difference in the microsomal PC/PE ratio could also be a factor influencing membrane fluidity (28). It has been shown that increasing the PC/PE ratio, through an increase in the transformation of PE to PC by methyltransferase, increases membrane fluidity (28). Results in Figure 6A show that the fluorescence anisotropy is correlated, to a certain extent, with the PC/PE ratio (r = 0.800, $0.05 < P < 0.1$), suggesting that decreased microsomal membrane fluidity might be associated with an increase in the PC/PE ratio. This finding is apparently opposite to what has been reported (28). In our study, however, the dietary protein source affected liver microsomal properties in a manner similar to that reported by Garda and Brenner (26) in an *in vitro* CHOL-supplementation study. In this case, it was suggested that CHOL-supplementation had no effect on the PC/PE ratio (9,29,30), but increased the CHOL-content, the CHOL/PL ratio and Δ6 desaturase activity in the microsomal preparation (26,31). Since our results did not show any correlation between the PC/PE ratio and Δ6 desaturase activity (Fig. 6B), we conclude that the change in the PC/PE ratio is not associated with the change in desaturase activity.

In summary, our results suggest that dietary protein modulates microsomal CHOL levels, the CHOL/PL ratio and membrane fluidity, and subsequently the activity of Δ6 desaturase (and possibly Δ5 desaturase) in liver microsomes.

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COMMUNICATION

Sterols of *Chaetoceros* and *Skeletonema*[1]

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Dietary sterol is required by the oyster for growth, and sterol is believed to be obtained primarily from dietary phytoplankton. Seven isolates of *Chaetoceros* and one of *Skeletonema*, which are of potential use as oyster food, were analyzed for sterol composition using gas chromatography, high-performance liquid chromatography and gas chromatography/mass spectrometry. *Skeletonema* and five isolates of *Chaetoceros* contained cholesterol as their major sterol. Two other isolates of *Chaetoceros* also contained cholesterol, but 24-methylenecholesterol was the principal sterol. Cholesterol has rarely been reported as the major sterol from phytoplankton. In view of the widespread occurrence of *Skeletonema* and *Chaetoceros* in the marine environment, these algae could be an important source of the oyster's cholesterol.
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The American oyster *Crassostrea virginica* has a complex sterol composition of some forty compounds (1–3). This complexity has been attributed to dietary sterols that are accumulated due to the oyster's inability to synthesize sterols from simple precursors (4–7). Oysters must therefore obtain sterols from their diet of phytoplankton. In carefully controlled studies, the growth rates of oysters have been correlated with several dietary factors, one of which is sterol composition (8). The principal sterol of the oyster is cholesterol (1,9); however, cholesterol is rarely found in phytoplankton species, which are used in the laboratory or in commercial hatcheries to feed oysters or clams (10). This leads to the conclusion that other as yet unexamined phytoplankton must provide the oyster's cholesterol. In studies designed to evaluate phytoplankton as food for juvenile oysters, Enright *et al.* (11,12) listed three species of *Chaetoceros* and *Skeletonema costatum* as the most effective phytoplankton for promoting growth of the juvenile oyster, *Ostrea edulis*. *S. costatum* was reported by Ballantine *et al.* (13) to contain 24-methylenecholesterol as the principal sterol, and it also contained cholesterol, 24-methylcholesterol and 24-ethylcholesterol in amounts greater than 10% of total sterol. In *Chaetoceros simplex calcitrans* the principal sterols were cholesterol and 24-methylenecholesterol (14), but in an unidentified *Chaetoceros* sp., 24-methylcholesterol and 24-ethylcholesterol were the principal sterols (15). In view of the importance of sterols to the oyster, and the reports of cholesterol in some isolates of the cosmopolitan marine genera *Chaetoceros* and *Skeletonema*, the present study was undertaken to quantitatively determine the sterol composition of all *Chaetoceros* and *Skeletonema* isolates available to us.

[1]Mention of trade names does not imply endorsement.

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

MATERIALS AND METHODS

Phytoplankton strains were obtained from the Milford Culture Collection (Strain number is listed in parenthesis in Table 1.). Five of the eight strains were bacterized, and three were axenic. Of the seven *Chaetoceros* isolates analyzed, only B-13 forms short chains (*ca.* 4 cells); the others grow as essentially solitary unicells with sizes easily consumed by post-set oysters (<10 μm). The *Skeletonema* strain produced only short chains (*ca.* 3–4 cells). Algae were cultured in enriched natural seawater medium "E" formulation (16) under aseptic conditions (17). Algae from the stationary phase were centrifuged, lyophilized and stored in a freezer before analysis. Cells from the centrifuged concentrate were diluted and counted in an Improved Neubauer Hemocytometer (American Optical Co., Buffalo, NY) with a microscope. Dry weights were determined by weighing a known number of cells collected on a glass fiber filter (Whatman, GF/F, Whatman Labsales, Hillsboro, OR), washed with ammonium formate isotonic to the growth medium and dried in an oven at 80°C. Means of two dry weight determinations were calculated for each species, ranges never exceeded 5% of the mean.

Lyophilized samples were extracted overnight in a Soxhlet apparatus with $CHCl_3$/methanol (2:1, vol/vol) and sterol ester, sterol glycoside and free sterol fractions were isolated by Biosil A column chromatography and analyzed by capillary gas chromatography (GC), as described previously (17). Sterols were identified by capillary GC and gas chromatography/mass spectrometry (GC/MS) (17). High-performance liquid chromatography (HPLC) was employed to assign C-24 stereoconfiguration.

RESULTS AND DISCUSSION

Cholesterol, desmosterol, 24-methylenecholesterol, 24-methylcholesterol, 24-ethylcholesterol, fucosterol and isofucosterol were significant components in the sterol fractions of the isolates studied. They were identified on the basis of their relative retention times in capillary GC as compared to authentic compounds and by analysis with capillary GC/MS (21).

Table 1 shows the free sterol composition of the phytoplankton examined. Ester sterol and glycoside sterol each made up less than 2% of the free sterol and were not examined further.

The sterol composition of *C. gracile* (isolates Chaet B and ARC-11), *C. muelleri* and *C. sp.* (NRAC-Chaet) were essentially identical, with cholesterol being slightly more

TABLE 1

Sterols of *Chaetoceros* and *Skeletonema*

Isolate	Dry wt μg/g	fg/cell	Sterol composition[a] (% of total sterol)						
			CHOL	DES	24MEC	24MC	24EC	FUCO	ISOFUCO
Chaetoceros gracile (Chaet B)	9.9	41	47		9			39	1
C. gracile (ARC-11)	10.1	40	49		6			33	3
C. muelleri (CHGRA)	14.4	69	48	9				41	1
C. sp. (NRAC-Chaet)	27.7	84	47		7	3		37	3
C. simplex (Chaet-G)	3.8	104	40	1	24	1		31	1
C. sp. (B-13)	3.7	51	1		35	20	23		10
C. calcitrans (Chaet-cal)	0.3	3	39	3	56				
Skeletonema costatum (NRAC-Skel)	2.0	16	71		8	3	12		1

[a]Abbreviations: CHOL, cholesterol; DES, desmosterol; 24MEC, 24-methylenecholesterol; 24MC, 24-methylcholesterol; 24EC, 24-ethylcholesterol; FUCO, fucosterol; ISOFUCO, isofucosterol.

abundant than fucosterol. Small amounts of 24-methylenecholesterol and isofucosterol were also present. The amount of sterol in three of these isolates on a per cell basis (40–84 fg/cell) was also similar, although NRAC Chaet contained appreciably more sterol on a dry weight basis (27.7 μg/g) than the other strains in this group. Cholesterol made up 40% of the sterols of *C. simplex*, but substantial quantities of both 24-methylenecholesterol and fucosterol were also present. The amount of sterol per cell in *C. simplex* (104 fg/cell) was slightly greater than in the other isolates examined (Table 1); however, cells of this strain were much larger than the others, resulting in % dry weight values less than those of the *C. gracile/muelleri* group. *Chaetoceros* sp. (B-13) is the only isolate examined that had large amounts of 24-ethylcholesterol and 24-methylcholesterol occurring. The latter sterol was examined by HPLC (18), which separates most 24α from 24β sterols. It revealed that the 24-methylcholesterol of *Chaetoceros* sp. (B-13) was all 24β-methylcholesterol (5-ergostenol). This system does not resolve 24-ethylcholesterol isomers. In each previous case where a member of the diatom order centrales was examined for stereochemistry at C-24, the β isomer was also reported (10). The sterols of *C. calcitrans* (Chaet-cal) differed from those in the other isolates in two respects. The total sterol was at least an order of magnitude less (3 fg/cell or 0.31 μg/g dry wt) in this isolate than in any other *Chaetoceros* isolate examined. Chaet-cal was the only isolate examined that did not contain C_{29} sterols. This isolate contained a small amount (3% total sterol) of desmosterol and the remaining sterol consisted only of cholesterol and 24-methylenecholesterol, with the latter sterol being most abundant. Cholesterol was prevalent in *S. costatum*, with the overall sterol content (16 fg/cell or 2.0 μg/g dry wt) being less than all *Chaetoceros* isolates except *C. calcitrans*. However, the second most abundant sterol in *Skeletonema* was the 24-ethylcholesterol and not fucosterol, as was the case in most *Chaetoceros* isolates (Table 1).

Previous indications (10) were that most algae in the families Chaetoceraceae and Thalassiosiraceae, to which *Chaetoceros* and *Skeletonema* belong, contain 24-methylenecholesterol as a principal sterol. The presence of 24-methylenecholesterol was demonstrated in each isolate examined in this study. The seven isolates of *Chaetoceros* examined showed four distinct compositional patterns. With the exception of *Chaetoceros* sp. (B-13), cholesterol was a substantial component, and was the prevalent component of six of these sterol mixtures. Cholesterol was also most abundant in *Skeletonema*. With the exception of the red algae (10), the prevalence of cholesterol in sterol mixtures is rare in most plant groups (22). Until now few phytoplankton were known to contain cholesterol, and it appeared that cholesterol in natural populations of oysters must come from dealkylation of dietary phytoplankton sterols. Rapid growth of oysters on cultured phytoplankton species that contain no cholesterol (8) suggests that dealkylation of certain phytosterols to cholesterol is likely to occur. Nevertheless, the discovery of the prevalence of cholesterol in these widely occurring marine phytoplankton isolates could point the way to locating direct phytoplankton sources of cholesterol for the oyster and other marine invertebrates in nature.

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Pollen Sterols from Three Species of Sonoran Cacti

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Cactus bees are important pollinators that contribute to the long-term stability of arid regions in the United States. Since all insects are dependent upon a dietary source of sterol for normal growth, development and reproduction, a study was undertaken to determine neutral sterols available to cactus bees. The total neutral sterol composition of hand-collected pollen was determined for three species of Sonoran cacti by gas-liquid chromatography and mass spectrometry. 24-Methylenecholesterol was the predominant pollen sterol in Engelmann's prickly pear, *Opuntia phaeacantha*, cholla, *O. versicolor*, and cardon, *Pachycereus pringlei*. Two pentacyclic triterpene alcohols, lupeol and moretenol, were also isolated. Since no cholesterol was detected in any of the pollen samples, cactus bees would have to utilize the 24-alkyl sterols unchanged or convert these sterols to cholesterol *via* dealkylation.
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Insects, unlike mammals and plants, are unable to synthesize sterols and therefore require a dietary source of sterol for normal growth and development (1). Many species of insects are capable of converting dietary C_{28} and C_{29} phytosterols to cholesterol, while other species lack this capability (2–4). Amongst the Hymenoptera, it appears that the phytophagous members of this group lack the ability to dealkylate plant sterols since the body sterols of these species examined contain little or no cholesterol (4–8). *Diadesai rinconis* Cockerell is a solitary cactus bee that feeds primarily on the pollen of *Opuntia* (9) and is therefore considered an important pollinator of these Sonoran cacti. The purpose of this investigation was to examine the neutral sterol content of several species of Sonoran cacti to determine the dietary sterols available to *Diadasia* bees.

MATERIALS AND METHODS

Isolation of sterols. Pollen from Engelmann's Prickly Pear cactus, *Opuntia phaeacantha*, from Cholla cactus, *O. versicolor*, and from Cardon cactus, *Pachycereus pringlei*, were hand-collected near Tucson, AZ, and stored in methanol at −20°C until workup. Each sample (2–10 g) was saponified under reflux using 4% KOH in a solution of ethanol/benzene/water (10:1:1, by vol). After 5 h, the solution was allowed to cool, acidified with 6N HCl, and subsequently extracted with hexane (3×) and diethyl ether (1×). The combined organic phases were dried *in vacuo* and fractionated over Florisil (Fisher Scientific, Fair Lawn, NJ) in a diethyl ether/hexane system as previously described (10). Fractions were monitored by thin-layer chromatography (TLC) and capillary gas-liquid chromatography (GLC). Sterols were purified prior to mass spectrometry by reversed-phase high-performance liquid chromatography (HPLC).

Analyses and instrumentation. All solvents for extraction and purification were reagent grade, redistilled. Solvents for HPLC were obtained from Burdick & Jackson (Baxter Scientific, Columbia, MD). TLC was done on high-performance silica gel 60 F_{254} plates developed in hexane/diethyl ether/acetic acid (60:40:1, by vol). Reversed-phase HPLC was performed on a Shandon ODS Hypersil column (4.6 × 250 mm, 5 μm particle size; Shandon Scientific, Runcorn, Chesire, United Kingdom) eluted with 98% aqueous methanol at 1 mL/min. Effluent was monitored at 200 and 215 nm using a Waters 991 photodiode array detector (Milford, MA). Capillary GLC was performed at 245°C on a Shimadzu GC-9A gas chromatograph (Columbia, MD) equipped with a J&W DB-1 fused silica column (15 m × 0.25 mm; 0.25 μm film; J&W Scientific, Folsom, CA) and a recording integrator. Mass spectra were obtained on a Finnigan 4500 gas chromatograph/mass spectrometer fitted with a J&W DB-1 fused silica column (30 m × 0.32 mm; 0.25 μm film) and temperature programmed (230°C for 2 min, then increased 5°C/min to 255°C and held). Electron impact spectra were collected at 70eV and a source block temperature of 150°C. All sterol identifications were based on relative retention time (to cholesterol) and mass spectra.

RESULTS

The predominant pollen sterol in all three cactus samples was 24-methylenecholesterol, with additional C_{28}, C_{29} and C_{30} sterols comprising the remainder. 24-Methylenecholesterol accounted for 80.3% of the neutral sterols in Prickly Pear to over 90% in Cardon (Table 1). The next most prevalent sterols were 24-ethylcholesterol (7.3%) and cycloartenol (7.2%) in Prickly Pear, fucosterol (6.6%) and 24-ethylcholesterol (5.6%) in Cholla, and 31-norcycloartenol (3.8%) and fucosterol (2.0%) in Cardon. Seven sterols, ranging in concentration from 0.4–2.2%, were found in only a single pollen source (Table 1). In addition, two pentacyclic triterpene alcohols were identified during this study. Lupeol was found in both the Prickly Pear and Cardon pollens, and mortenol [a'-neogammacer-22(29)-en-3β-ol] was isolated from Prickly Pear. No cholesterol was detected in any of the cactus pollens examined.

DISCUSSION

One species of cactus, the saguaro, *Cereus giganteus* (=*Carnegiea giganteus*) was included in the survey of pollen sterols by Standifer and collaborators (11) and was shown to contain predominantly (94%) 24-methylenecholesterol. The preponderance of 24-methylenecholesterol in saguaro pollen was later confirmed by Nes and Schmidt (12), who reported several additional sterols including 24-dehydropollinasterol and a new sterol, 25(27)-dehydrolanost-8-enol. The only parallels we can perhaps draw between these two as well as our present study are with a close relative of saguaro, cardon (*Pachycereus pringlei*), where we found the pollen to contain more than 90% 24-methylenecholesterol.

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

TABLE 1

Relative Percentages of Neutral Sterols from Sonoran Cactus Pollen[a]

	Prickly Pear *(Opuntia phaeacantha)*	Cholla *(Opuntia versicolor)*	Cardon *(Pachycereus pringlei)*
Pollinastanol	—	0.7	—
24-Dehydropollinastanol	0.8	—	—
24-Methylenecholesterol	80.3	81.2	90.3
24-Methylcholesterol	0.4	1.1	—
Ergosta-8(9),24(28)-dienol	—	—	1.3
24-Ethylcholesta-5,22-dienol	—	—	1.1
31-Norcycloartenol	1.8	—	3.8
24-Ethylcholesta-8,24(28)-dienol	—	—	0.4
24-Methylenepollinastanol	—	1.0	—
24-Ethylcholesterol	7.3	5.6	0.3
Fucosterol	—	6.6	2.0
Cycloeucalenol	2.2	—	—
Cycloartenol	7.2	2.0	0.4
Unknown	—	1.8	0.4

[a]Sterols were identified by a combination of capillary gas-liquid chromatography (GLC) and capillary gas chromatography/mass spectrometer. Relative percentages were determined by integration of the flame-ionization detector signal from capillary GLC.

Though no literature appears to exist on sterols from *Opuntia* pollen, several studies involving other plant parts from this genus are interesting. Examination of mature photosynthetic tissue of *O. comoduensis* and *O. humifusa*, two nonsonoran species, indicated that sitosterol was the primary sterol, accounting for 86.7 and 87.0%, respectively (13). The sterol composition of the fruits of seven species of *Opuntia*, including *O. phaeacantha*, indicated that sitosterol comprised anywhere from 50.7 to 87.7%. In Engelmann's Prickly Pear, 81% of the neutral sterols consisted of sitosterol (14). Based on our results, it is apparent that the sterol content of pollen can be markedly different from that of the tissue since 24-ethylcholesterol (presumably sitosterol) accounted for only 7.3% of the sterols in prickly pear pollen, and only 5.6% in the pollen of cholla (*O. versicolor*).

It will be of great interest to examine the sterol content of the solitary bees that frequent specific pollen sources, given none of the pollen sources in the present study contain even trace amounts of cholesterol. The role of plant sterols in determining how different species of *Drosophila* utilize different species of cacti for feeding and breeding is well documented (15,16, and references therein). It remains to be determined if cactus bees exhibit a similar dependence on specific, dietary sterols.

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Changes in Fatty Acid Desaturation in Hepatic and Intestinal Tissues Induced by Intestinal Resection

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We have studied the activities of Δ9, Δ6 and Δ5 desaturases in rat liver and jejunum when the entero-hepatic circulation was interrupted by either 50 or 75% intestinal resection. Desaturase activities in caecal mucosa were also determined in control and operated rats. Distal small bowel resection significantly altered the activities of desaturase enzymes in liver, jejunum and caecum. Thus, the Δ9 desaturase activity was lower in hepatic microsomes from operated animals, and this decrease was not related to the extent by which the intestine was removed. However, a significant increase in both Δ6 and Δ5 desaturases was found in these animals compared to sham-operated rats, the increase in both desaturases being higher after 75% than after 50% intestinal resection. The activities of Δ9 and Δ5 desaturases were significantly increased in jejunal mucosa of resected rats. The activity of Δ6 desaturase was increased only in 50% resected-animals. An increase in Δ6 desaturase activity was observed in caecal mucosa after resection, together with a decrease in Δ9 desaturase and no change in Δ5 desaturase activities. Enzyme activities were highest in the liver relative to the activity in jejunal and caecal homogenates. In sham rats, the caecal mucosa showed higher Δ9 and Δ6 but lower Δ5 desaturase activities than in jejunal mucosa.

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It is well known that intestinal resection, by altering the entero-hepatic circulation (EHC), decreases the input of bile acids into the liver, leading to changes in both hepatic (1) and intestinal (2) lipid metabolism. Thus, the hepatic and intestinal activities of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and acyl-CoA:cholesterol acyltransferase (ACAT), the major rate-controlling enzymes in cholesterol biosynthesis and conversion of cholesterol into cholesteryl esters, respectively, are modified after the interruption of the EHC (3-5). The relative availability of saturated and unsaturated fatty acids for phospholipid synthesis is determined by the diet and the activity of elongase and desaturase enzymes (6). Since changes in both lipid composition and the fatty acid profiles of hepatic and intestinal phospholipid fractions are also observed after the interruption of the EHC (2,5), it is quite likely that changes in desaturase activities might occur after this operation.

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Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; ANOVA, analysis of variance; ATP, adenosine 5'-triphosphate; DSBR, distal small bowel resection; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; EHC, entero-hepatic circulation; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HMG-CoA, hydroxymethylglutaryl-coenzyme A; NADH, nicotinamide adenine dinucleotide (reduced form); TLC, thin-layer chromatography.

Δ9, Δ6 and Δ5 desaturase enzymes have been well documented in liver microsomes of several mammalian species (7) and, recently, they have been found in the mucosa of jejunum and ileum (8). However, as far as we are aware, desaturase activities in the caecal mucosa have not been previously identified. Our study was undertaken in order to determine the activities of desaturase enzymes bringing about the conversion of fatty acids in rat liver and jejunum when the EHC was interrupted by either 50 or 75% distal small bowel resection (DSBR). In addition, desaturase activities in caecal mucosa were determined in control and operated rats.

MATERIALS AND METHODS

Animals. Male Wistar-strain rats, purchased from Iffa-Credo (Lyon, France) and weighing 300 g each, were used. The animals were given food (Panlab A-04, Barcelona, Spain) and water *ad libitum* and housed in a room maintained at 21 ± 2°C with lights on from 0800 to 2000. The composition of the diet was as follows: lipids, 3.5%; protein, 19.0%; starch, 66.0%; non-nutritive cellulose, 5.0%; mineral mix, 5.5%; and vitamin mix, 1.0%. The fatty acid composition of the diet is shown in Table 1. The rats were randomly assigned to one of three groups: sham operated, 50 and 75% DSBR. Operative details have been previously described (9). Briefly, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbitone (4.5 mg/100 g body weight) after a 24-h fasting period, laparotomy was performed and rats assigned for DSBR underwent either 50 or 75% DSBR by excision of the distal small intestine beginning 1 cm proximal to the ileocaecal junction. Before DSBR, the blood vessels of the resected intestinal segment were tied and sectioned, and the blood supply and the innervation of the remaining intestine were carefully maintained. Intestinal continuity

TABLE 1

Fatty Acid Composition of the Diet (%, wt/wt)[a]

Fatty acids	Composition (%, wt/wt)
16:0	18.0
16:1n-7	2.3
18:0	4.0
18:1n-9	20.7
18:2n-6	49.4
18:3n-3	3.4
20:0	0.1
20:1n-9	1.6
22:0	0.3
Total saturated	22.1
Total monounsaturated	24.6
Total n-6	49.4
Total n-3	3.4

[a]See Materials and Methods.

was re-established by an end-to-end anastomosis. Finally, both muscle and cutaneous layers were sutured separately with appropriate thread. Rats from the sham-operated group underwent simple mid-small intestinal transection, without removal of any tissue, followed by re-anastomosis. After the surgical operation, the rats were housed in a temperature-controlled laboratory with a strict 0400–1600/1600–0400 dark/light cycle. At 6 wk after DSBR, the rats were fasted overnight (with access to water only) and killed by stunning and cervical dislocation.

Sample preparation procedure. After the animals were killed, the abdomen was opened and livers, jejunum and caecum were removed from each rat and placed in ice-cold physiological saline. All subsequent operations were done at 4°C. Livers were homogenized in a Potter-Elvehjem homogenizer with ice-cold homogenization medium containing 0.25 M sucrose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 20 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) and 5 mM dithiothreitol (DTT). Each homogenate was centrifuged for 20 min at 15,000 × *g*. The supernatant was collected and centrifugation (15,000 × *g*) was repeated. The 15,000 × *g* supernatant was centrifuged at 105,000 × *g* for 60 min in a Sorvall (Newton, MA) ultracentrifuge, model OTD 50B. The resulting microsomal pellets were immediately frozen in liquid N_2 and stored at −70°C until assayed. The mucosal surface of jejunum and caecum was removed by gently scraping with a microscope slide and stored at −70°C until further use. Jejunal and caecal mucosal scrapings were homogenized in 0.25 M sucrose buffer containing 10 mM HEPES (pH 7.4), 20 mM EDTA, 2 mM EGTA and 5 mM DTT. Portions of jejunal and caecal homogenates were used for desaturase assays. Desaturase activities of both liver microsomes and intestinal mucosas were not affected by the storage time (results not shown).

Assay of desaturases. The hepatic microsomes and jejunum and caecum mucosas were assayed for Δ9, Δ6 and Δ5 desaturase activities as previously described (10), with minor modifications. The assay mixture contained, in a final volume of 1 mL, 1.25 mM NADH, 1.5 mM reduced glutathione, 5 mM ATP, 5 mM $MgCl_2$, 0.3 mM coenzyme A and 75 μM [1-^{14}C]palmitic, 75 μM [1-^{14}C]linoleic or 75 μM [1-^{14}C]eicosa-8,11,14-trienoic acid in the form of the sodium salt-albumin complex (1 μg free fatty acid/11.5 μg bovine serum albumin). Incubations were done at 37°C for 20 min in a metabolic shaker. Enzyme assays were terminated by addition of 1 mL of 10% (wt/vol) KOH in ethanol, followed by saponification at 100°C for 30 min and addition of 1 mL of 4 N H_2SO_4 under nitrogen atmosphere to avoid oxidation of fatty acids. Lipids were extracted twice with 5 mL chloroform/methanol (2:1, vol/vol). The organic phases were combined and evaporated under nitrogen. Fatty acids were methylated using 14% boron trifluoride in methanol. Fatty acid methyl esters were separated on commercial silica gel G-60 thin-layer chromatography (TLC) plates containing 5% (wt/vol) $AgNO_3$. Plates were developed three times at 4°C in benzene, and radioactivity was analyzed in a Berthold (Nashua, NH) Automatic TLC-Linear Analyzer with the aid of a computer program.

Protein assay. Microsomal and mucosal proteins were determined by the method of Lowry *et al.* (11) with bovine serum albumin as the standard. Enzyme activities were expressed as pmol of desaturated product formed per min per mg of either microsomal (liver) or mucosal (jejunum and caecum) protein.

Statistical analysis. Results are presented as the mean ± standard deviation. An analysis of variance as a first step prior to the use of *t*-test was used to test the significance of the difference between the means for sham-operated and resected animals.

RESULTS AND DISCUSSION

A significant decrease in the hepatic activity of Δ9 desaturase was observed after the interruption of the EHC. However, an increase in both Δ6 and Δ5 desaturase activities was found in these animals compared to sham rats (Table 2). Δ6 Desaturase converts 18:2n-6 into 18:3n-6 fatty acid. Increased Δ6 desaturase activity in rat liver microsomes of operated rats should increase the conversion of 18:2n-6 to 18:3n-6. We have previously reported that γ-linolenic acid (18:3n-6) levels were increased in hepatic microsomes of resected rats and that the increase was higher after 75% than after 50% DSBR (5). Thus, the relatively high content of 18:3n-6 in operated rats might be related to enhanced Δ6 desaturase. Similarly, the levels of 20:3n-6, the substrate for Δ5 desaturase, increased in hepatic microsomes of 75%-resected rats (5). The elevation in 20:3n-6 together with a subsequent decrease in arachidonic acid observed after massive resection (75%) suggests an inhibition of Δ5 desaturase activity. However,

TABLE 2

Effect of the Interruption of the Entero-Hepatic Circulation on Δ9, Δ6 and Δ5 Desaturase Activities in Hepatic Microsomal Membrane[a]

	Desaturase		
	Δ9	Δ6	Δ5
Sham-operated	286.6 ± 13.51	88.0 ± 08.5	148.3 ± 05.2
50%-DSBR	160.6 ± 04.2[b]	227.3 ± 06.2[b]	195.7 ± 08.7[b]
75%-DSBR	192.2 ± 20.0[b,c]	479.0 ± 13.1[b,e]	228.1 ± 07.1[b,d]
Results of ANOVA ($P <$)	0.0001	0.0001	0.0001

[a]The data (expressed as pmol/min/mg microsomal protein) are presented as the mean ± SD of five animals. DSBR, distal small bowel resection; ANOVA, analysis of variance.

[b]$P < 0.001$ 50 or 75% resected rats *vs.* sham animals.

[c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.001$ 75% resected rats *vs.* 50% resected animals.

TABLE 3

Effect of the Interruption of the Entero-Hepatic Circulation on Δ9, Δ6 and Δ5 Desaturase Activities in Homogenates of Jejunal and Caecal Mucosas[a]

	Desaturase		
	Δ9	Δ6	Δ5
Jejunum			
Sham-operated	14.8 ± 0.35	14.0 ± 2.30	20.3 ± 0.91
50%-DSBR	32.1 ± 0.75[d]	18.3 ± 1.92[b]	27.3 ± 3.42[c]
75%-DSBR	28.5 ± 0.43[d,e]	16.7 ± 1.54	45.2 ± 1.95[d,e]
Results of ANOVA ($P <$)	0.0001	0.0280	0.0001
Caecum			
Sham-operated	34.6 ± 1.12	19.2 ± 0.67	15.6 ± 0.53
50%-DSBR	27.5 ± 0.87[d]	27.7 ± 0.94[d]	15.1 ± 0.57
75%-DSBR	18.5 ± 0.58[d,e]	41.2 ± 1.83[d,e]	14.5 ± 1.33
Results of ANOVA ($P <$)	0.0001	0.0001	0.1896

[a]The data (expressed as pmol/min/mg mucosal protein) are presented as the mean ± SD of five animals. Abbreviations as in Table 2.

[b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$ 50 or 75% resected rats *vs.* sham animals.

[e]$P < 0.001$ 75% resected rats *vs.* 50% resected animals.

our *in vitro* results for Δ5 desaturase activity showed an increase in 75%-resected animals (Table 2). No significant changes in the levels of both 20:3n-6 and 20:4n-6 had previously been observed in 50%-resected rats (5), although an increase in Δ5 desaturase was found in hepatic microsomes of these animals (Table 2). Therefore, *in vivo* metabolism cannot always be surmised from *in vitro* observations, since there are many factors *in vivo* that would not be operative under *in vitro* conditions.

Consistent with previous reports (8,12), the present study shows that the intestinal mucosa is capable of desaturating palmitic acid (16:0), linoleic acid (18:2n-6) and eicosatrienoic acid (20:3n-6) and that this desaturation is affected by the interruption of the EHC. Thus, the activities of the Δ9 and Δ5 desaturase enzymes were significantly increased in jejunal mucosa as a consequence of the interruption of the EHC, the increase in Δ5 desaturase activity being related to the extent of the EHC interrupted. However, the activity of Δ6 desaturase was increased only after 50% DSBR (Table 3).

As far as we are aware, desaturase activities of caecal mucosa have not been previously determined. Table 3 shows the activities of Δ9, Δ6 and Δ5 desaturases in caecal mucosa of sham-operated and resected rats. The intestinal resection produced in caecal mucosa a decrease in Δ9, an increase in Δ6 and no change in Δ5 desaturase activities (Table 3).

In sham rats the caecal mucosa showed higher Δ9 and Δ6 desaturase activities than jejunal mucosa. Δ5 Desaturase activity was, however, lower in caecal mucosa (Table 3). The observation that the levels of all three desaturase enzymes were higher in liver than in intestine is consistent with previous results (8). However, the differences in enzymatic activities between liver and intestine might be due, in part, to the different preparations used, microsomes for liver and mucosal homogenates for intestine.

The results of this study show that DSBR significantly altered the activities of Δ9, Δ6 and Δ5 desaturase enzymes in liver, jejunum and caecum but in different ways. Several factors could explain the current observations. Thus, it is well known that the interruption of the EHC causes lipid malabsorption (13). This malabsorption is due to an effective loss of absorptive surface area together with a defect in the EHC of bile salts. Therefore, an effect of intestinal resection on the dietary source of liver fatty acids cannot be ruled out. In addition, if the intestinal mucosa is capable of desaturating fatty acids, then loss of intestinal tissue by DSBR might also produce changes in fatty acid metabolism.

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Garlic Supplementation and Lipoprotein Oxidation Susceptibility

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Interventions which make serum lipoproteins less susceptible to oxidation may be antiatherogenic. The antioxidant properties of garlic which have been demonstrated *in vitro* led us to investigate the effects of garlic supplements on lipoprotein oxidation susceptibility in humans. Ten healthy volunteers were given 600 mg/d of garlic powder (6 tablets of Kwai®) for two weeks in a placebo-controlled, randomized, double-blind crossover trial. We found that although serum lipid and lipoprotein levels were not lowered in this short time period, the *ex vivo* susceptibility of apolipoprotein B-containing lipoproteins to oxidation was significantly decreased (−34%). Because garlic has been reported to beneficially affect serum lipid levels, platelet function, fibrinolysis and blood pressure, this additional effect of retarding lipoprotein oxidation may contribute to the potential antiatherosclerotic effect of garlic.
Lipids 28, 475–477 (1993).

There is growing evidence that the oxidation of low density lipoproteins (LDL) may play a significant role in the development of atherosclerosis (for a review, see Ref. 1). Accordingly, interventions which can prevent the oxidation of LDL may be expected to slow atherogenesis, reducing the incidence of coronary heart disease, possibly even without lowering serum LDL levels.

There are currently three nutrients (ascorbic acid, α-tocopherol, monounsaturated fatty acids, Refs. 2–4), one food preservative (butylated hydroxytoluene, Ref. 5) and one drug (probucol, Ref. 6) that function as *in vivo* antioxidants. The latter two have also been shown to be antiatherosclerotic in animal models (5,6). Garlic is a food which has has been reported to lower cholesterol levels (7), and in addition, to have a beneficial effect on a number of other physiological disorders contributing to heightened cardiovascular risk, *e.g.,* high blood pressure (8), enhanced platelet aggregation (9–11), delayed fibrinolysis (9) and vasoconstriction (9). A recent report suggested that garlic powder extracts also act as antioxidants *in vitro* (12). Therefore, we conducted a trial in humans to determine the effects of short-term garlic supplementation on plasma lipid levels and on lipoprotein oxidation susceptibility (LOS).

MATERIALS AND METHODS

Ten healthy, normolipidemic subjects, five males and five females (mean age 32 ± 10 years) volunteered for the study. The subjects were taking no medications known to affect serum lipids, and none were taking garlic or significant quantities of antioxidants (vitamins C or E or β-carotene) in their background diets. Subjects were asked to keep their smoking and exercise patterns constant throughout the study. Informed consent was obtained from each subject prior to beginning the study, which had been approved by the Human Subjects Committee of the University of Kansas Medical Center (Kansas City, KS).

The subjects were instructed to take six 100-mg tablets of garlic powder a day for two weeks (Kwai®, Lichtwer Pharma GmbH, Berlin, Germany) or placebo. The study had a randomized, placebo controlled, double-blind crossover design. There was a one-week washout period between the two treatment periods.

Maintaining dietary stability was highly stressed throughout the study, and subjects were advised to avoid any foods containing significant amounts of garlic. Diets were monitored by having the subjects complete two three-day diet diaries (one during each treatment period) which were analyzed by Professional Nutrition Systems, Inc. (Kansas City, KS). This was done to determine whether the changes in plasma lipid levels, if any, were due to the garlic or to a change in diet. Results of these analyses were expressed as the RISCC rating (ratio of ingested saturated fat and cholesterol to calories) which summarizes the major cholesterol raising nutrients in the diet as a single numerical score. An RISCC rating of 20–24 is typical of the American diet (13).

Blood samples were drawn (always after a 12-h overnight fast) at the beginning and the end of each test period. Lipids and lipoproteins in plasma containing ethylenediaminetetracetic acid (1 mg/mL) were analyzed for cholesterol and triglycerides using enzymatic methods on a Cobas Mira (Roche Diagnostics, Belleville, NJ). The plasma high density lipoprotein (HDL) cholesterol levels were measured following precipitation of the apoprotein B (apoB)-containing lipoproteins with heparin/manganese chloride. LDL cholesterol was estimated by the Friedwald equation (14). These methods have been described in detail elsewhere (15), and were carried out in our laboratory which participates in the Lipid Standardization Program of the Centers for Disease Control (16).

LDL phospholipid and cholesteryl ester fatty acid composition. Lipids were extracted from LDL isolated from plasma by sequential ultracentrifugation between densities 1.019 and 1.063 kg/L. Thin-layer chromatography was used to separate the various lipid classes, and the cholesteryl ester and phospholipid bands were obtained. The lipids were transmethylated with boron trifluoride and analyzed by gas-liquid chromatography in a GC9A Gas Chromatograph (Shimadzu Corp., Columbia, MD), equipped with a 30-m, 0.32 mm i.d., SP2330 capillary column. These methods were described in detail previously (15).

LOS test. A 500-μL plasma sample was treated with 50 μL of a solution containing 0.2 mM dextran sulfate (MW 50,000; Genzyme, Cambridge, MA) and 0.5 M $MgCl_2 \cdot 6H_2O$ to precipitate the apoB-containing lipoproteins [LDL and very low density lipoproteins (VLDL)]

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Abbreviations: apoB, apolipoprotein B-100; HDL, high density lipoproteins; LDL, low density lipoproteins; LOS, lipoprotein oxidation susceptibility; MDA, malondialdehyde; RISCC, ratio of ingested saturated fat and cholesterol to calories; TBA, thiobarbituric acid; TBARS, TBA reactive substances; TCA, trichloroacetic acid; VLDL, very low density lipoproteins.

according to Bachorik and Albers (17). After centrifugation at 3,000 rpm at 20°C for 10 min, the supernatant was removed, and 1 mL of 6% bovine serum albumin and another 50 μL of the dextran sulfate magnesium solution was added. The solution was briefly vortexed and recentrifuged as above to wash away any HDL or residual serum proteins (except, of course, albumin). The supernatant was removed and the washed precipitate (containing LDL and VLDL) was dissolved in 2.5 mL of 4% NaCl. A volume of redissolved precipitate containing 100 μg of non-HDL cholesterol was combined with sufficient 4% NaCl to give a total volume of 500 μL (approximately a 1:5 dilution). Fifty μL of a 0.5 mM $CuCl_2 \cdot 2H_2O$ solution was added (final copper concentration was 46 μM), and then the samples were incubated at 37°C in a shaking water bath for 3 h. Next, thiobarbituric acid reactive substances (TBARS) were measured (18) by adding 2 mL of the TBARS reagent to each tube. [This reagent contained 26 mM thiobarbituric acid (TBA) and 0.92 M trichloroacetic acid (TCA) in 0.25 N HCl. The TBA was added first, heated and stirred. After it was dissolved, the TCA was added, and the solution brought to volume with 0.25 N HCl. The reagent was stored in a dark bottle at room temperature.] The mixture was heated at 100°C in a waterbath for 15 min. After removing and cooling the tubes, 2.5 mL *n*-butanol was added, the tubes were vortexed, and then centrifuged for 15 min at 3,000 rpm at room temperature. The pink upper layer was removed and the optical density was determined in a spectrophotometer at 532 nm. A standard curve was constructed with malondialdehyde (MDA, 0.5–16 nmol/mL), and the results were expressed as nmol of MDA produced per mg of non-HDL cholesterol. The coefficient of variation of the method was 4% intraassay and 9% interassay. The mean LOS value for 20 normal subjects was 66 ± 22 nmol MDA/mg non-HDL cholesterol. Using this assay, we have shown that probucol treatment reduced LOS by 95%, while fish oil supplementation (high in polyunsaturated fatty acids) raised LOS by 45% (Harris, W.S., unpublished data).

Statistical evaluation. As the subjects served as their own control for four observations (pre and post, garlic and placebo), the data were analyzed by ANOVA with repeated measures followed by the Neuman-Kuels *post-hoc* test. A P value of <0.05 was required for statistical significance.

RESULTS AND DISCUSSION

Two weeks of garlic supplementation did not alter plasma total, LDL or HDL, cholesterol or triglyceride levels (Table 1). This was not a wholly unexpected finding as previous investigators had reported only small decreases in lipid levels after 4 wks, with maximum effects noted at 16 wks (7). Thus, longer periods of supplementation would probably be needed to change these parameters.

On the other hand, garlic supplementation significantly reduced the susceptibility of the apoB-containing lipoproteins to copper-induced oxidation (Fig. 1). LOS values went from 80 ± 25 to 53 ± 32 nmol MDA/mg non-HDL cholesterol, a 34% decrease ($P < 0.05$). Values after two weeks of placebo were unchanged at 72 ± 19 *vs.* 73 ± 16 nmol MDA/mg non-HDL cholesterol. Only about half of the subjects actually experienced decreases in LOS, and two showed remarkable reductions. The relative non-responsiveness of the other half of the subjects suggests that other factors (dose, duration, endogenous antioxidants) may modulate the effectiveness of garlic.

Dietary stability was confirmed in this study by analysis of diet diaries during both phases. RISCC ratings were identical (23 ± 5) in both phases, indicating that the subjects were consuming a typical American diet, and that there was no change in background diets between periods which would be expected to alter plasma lipid levels. Because the diets were stable, the effects observed on LOS were likely to be due to the garlic powder supplements.

A change in LDL fatty acid composition (*i.e.*, a reduced amount of polyunsaturated fatty acids) could have been responsible for the reduced susceptibility of lipoproteins to oxidative stress during the garlic period. However, we found no change whatever in LDL phospholipid or cholesteryl ester fatty acid patterns (Table 2). Data on LDL vitamin E levels are not available, but because garlic powder contains no vitamin E, it seems unlikely that changes in α-tocopherol status would explain these findings.

Kourounakis and Rekka (12) recently tested the antioxidant properties of garlic powder, Kwai and alliin (the odorless precursor which is converted to odoriferous allicin when exposed to alliinase, an enzyme released when the

TABLE 1

Effects of Two Weeks of Kwai® Supplementation on Serum Lipids and Lipoproteins in Ten Healthy Volunteers (mg/dL)[a]

	Placebo		Garlic	
	Before	After	Before	After
Total cholesterol	174 ± 39	173 ± 38	176 ± 37	175 ± 39
Triglyceride	75 ± 22	78 ± 19	79 ± 21	79 ± 24
HDL-cholesterol	54 ± 7	55 ± 7	56 ± 6	56 ± 8
LDL-cholesterol	104 ± 36	103 ± 33	106 ± 34	104 ± 36

[a]HDL, high density lipoprotein; LDL, low density lipoprotein.

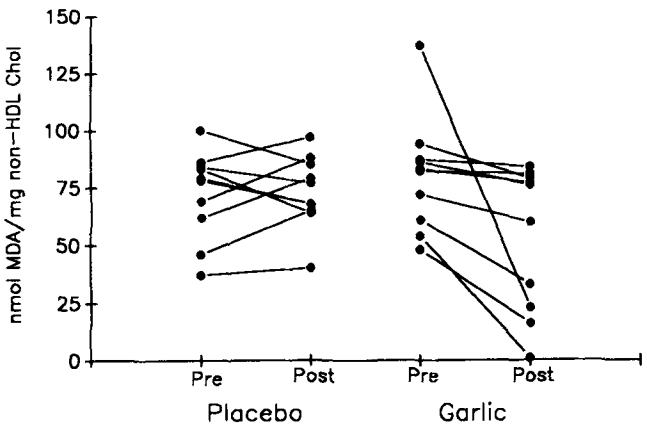

FIG. 1. Six garlic powder tablets (Kwai®) or placebo were given daily to ten healthy volunteers for two weeks in a randomized, crossover design. Lipoprotein oxidation susceptibility (LOS) was assessed (see Materials and Methods) pre and post dosing for both treatments. Individual results are presented here; mean LOS was reduced by 34% by Kwai treatment and increased by 1% by placebo ($P < 0.05$). HDL, high density lipoprotein; MDA, malondialdehyde.

TABLE 2

Saturated, Monounsaturated and Polyunsaturated Fatty Acid Composition of Low Density Lipoproteins Cholesteryl Esters and Phospholipids During Placebo and Garlic Phases (mol% of total fatty acids)[a]

	Phospholipids			Cholesteryl esters		
Period	Sat	Mono	Poly	Sat	Mono	Poly
Placebo	70 ± 5	7 ± 1	23 ± 5	26 ± 8	16 ± 3	58 ± 8
Garlic	68 ± 5	7 ± 2	24 ± 5	26 ± 11	16 ± 6	57 ± 8

[a]Sat, saturated; mono, monounsaturated; poly, polyunsaturated.

garlic clove is crushed). They reported that both the garlic powder and Kwai were effective antioxidants when tested against rat hepatic microsomes stressed by ascorbic acid/Fe^{2+} and evaluated by TBARS generation. The garlic powder and alliin were also tested for their ability to scavenge hydroxyl radicals. Only the powder was effective at concentrations between 0.8 and 3.3 mM.

The application of these results to the *in vivo* situation is unclear, at best, as blood levels of garlic powder constituents (alliin or allicin) in subjects taking the products have eluded quantitation. Garlic is rich in a variety of sulfur-containing compounds (diallyldisulfide, vinyldithiines, ajoene), which are all potential antioxidants, but finding the putative *in vivo* antioxidant may be difficult.

In view of these considerations, it was with some skepticism that we evaluated the antioxidant effects of Kwai in healthy volunteers, and it was also with some surprise that we discovered that some component of this garlic preparation appeared to retain its antioxidant effect *in vivo*, even protecting isolated lipoproteins from oxidative stress. Future studies will be needed to determine exactly which component is responsible.

We conclude that 600 mg of Kwai taken for only two weeks significantly decreased the lipoprotein oxidation susceptibility without altering serum cholesterol levels in normal adults. If confirmed, such an effect may be considered to be potentially antiatherogenic. Trials should be undertaken to examine this effect in larger groups, for longer periods of supplementation, and in patients at increased risk for heart disease. In addition, the effects of garlic on atherosclerosis-prone animal models should be evaluated.

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ARTICLE

Protein Kinase C-Dependent Stimulation of Phospholipase D in Phospholipase C-Treated Fibroblasts

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Treatment of [^{14}C]choline- or [^{14}C]ethanolamine-labeled NIH 3T3 fibroblasts with *Bacillus cereus* phosphatidylcholine-specific phospholipase C (PLC) enhanced phospholipase D (PLD)-mediated hydrolysis of the respective ^{14}C-labeled phospholipids. PLD activity was stimulated by 1.5 U/mL of PLC and by 100 nM of the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) to similar extents. Treatment of [^{14}C]palmitic acid-labeled fibroblasts with PLC in the presence of ethanol also enhanced PLD-mediated formation of phosphatidylethanol; the effects of PLC and PMA were nonadditive. PLC had no effect on PLD activity in fibroblasts in which PKC was down-regulated by prolonged (24 h) treatment with 300 nM PMA. These data indicate that treatment of fibroblasts with exogenous PLC results in PKC-dependent activation of PLD.
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Recent evidence suggests that phospholipase C [EC 3.1.4.3] (PLC)-mediated hydrolysis of phosphatidylcholine (PtdCho) may be involved in the regulation of cell growth by platelet-derived growth factor (1), *ras* proteins (2–4), insulin (2), colony stimulating factor (5), epidermal growth factor (6) and hepatocyte growth factor (7). In agreement with the proposed role of cellular PLC as a cell growth regulator, exogenous addition of *Bacillus cereus* PtdCho-hydrolyzing PLC to fibroblasts was found to elicit a potent mitogenic response (1).

The mechanism by which the action of PLC leads to increased cell growth is not known. The primary products of PtdCho hydrolysis are 1,2-diacylglycerol and choline phosphate. While choline phosphate is not known to be part of the cellular signalling system, 1,2-diacylglycerol is an activator of protein kinase C (PKC) (8,9). However, in fibroblasts in which PKC-α was down-regulated by prolonged treatment with phorbol 12-myristate 13-acetate (PMA), exogenous PLC remained mitogenic (1). On this basis, PKC is not presently thought to serve as a mediator of the growth regulatory effect of PLC (1).

In most cell types examined so far, activated PKC is a major regulator of phospholipase D [EC 3.1.4.4] (PLD) (10–12). This enzyme is capable of rapidly generating phosphatidic acid, a potent mitogen in fibroblasts and several other cell lines (13–19). Thus, in principle, activation of PLD could be a requirement for the growth stimulatory action of PLC.

Although exogenous PtdCho-hydrolyzing PLC is known to stimulate the phosphorylating activity of cellular PKC (20), this may not automatically lead to the activation of PLD because regulation of this latter enzyme by PKC appears to involve a nonphosphorylating mechanism (21). Furthermore, regulation of PtdCho synthesis by exogenous PLC, which is mediated by 1,2-diacylglycerol, was shown to occur by a PKC-independent mechanism (22). Here, we show that exposure of NIH 3T3 fibroblasts to PLC leads to enhanced, PKC-dependent hydrolysis of phospholipids by PLD.

MATERIALS AND METHODS

Materials. PLC from *B. cereus*, PMA and Dowex-50-W(H^+ form) were purchased from Sigma Chemical Co. (St. Louis, MO); [*methyl*-^{14}C]choline chloride (50 mCi/mmol), [2-^{14}C]ethanolamine (50 mCi/mmol) and [1-^{14}C]palmitic acid (60 mCi/mmol) were from Amersham (Arlington Heights, IL); and tissue culture reagents were bought from Gibco (Grand Island, NY). Phosphatidylethanol (PtdEtOH) was prepared by PLD-catalyzed reaction from PtdCho and ethanol as described earlier (23).

Treatment of [^{14}C]choline-prelabeled fibroblasts. NIH 3T3 fibroblasts were grown to ≈80–90% confluency in 12-well culture dishes in the presence of [*methyl*-^{14}C]choline (0.5 μCi/mL) for 48 h. Fibroblasts were washed twice and then incubated in fresh medium for another 3-h period (needed to lower intracellular levels of free [^{14}C]choline). Fibroblasts were then treated with PLC and/or PMA in the presence of 20 mM unlabeled choline (final vol, 0.3 mL) for 5–20 min as indicated. After treatments with 0.75 or 1.5 U/mL of PLC or 100 nM PMA for 20 min, 95–98% of cells were viable, determined by the Trypan Blue dye exclusion assay. Incubations were terminated by adding 1 mL of ice-cold methanol to the wells. The methanol extracts were transferred to tubes containing 2 mL chloroform. The wells were washed twice with 0.5 mL of methanol.

Treatment of [^{14}C]ethanolamine- or [^{14}C]palmitic acid-labeled fibroblasts. Fibroblasts were grown in 150 mm-diameter plastic dishes for 48 h in the presence of [2-^{14}C]ethanolamine (0.25 μCi/mL), or for 24 h in the presence of [1-^{14}C]palmitic acid (0.25 μCi/mL). Fibroblasts were washed and then incubated in fresh medium for 3 h (to decrease the cellular level of unincorporated radiolabeled precursors; see Refs. 23–25). Fibroblasts were harvested by gentle scraping from 2 to 4 dishes. Washed fibroblasts (0.9–1.1 × 10^6 cells/mL) were incubated (final vol 0.25 mL) in an incubator at 37°C in the presence of agents as indicated. In the case of [^{14}C]ethanolamine-labeled fibroblasts, the incubation medium contained 2 mM unlabeled ethanolamine to prevent metabolism of newly formed [^{14}C]ethanolamine (24,25). Incubations were terminated by the addition of 4 mL of chloroform/methanol (1:1, vol/vol).

Separation of ^{14}C-labeled hydrolytic products. PtdEtOH was separated from other phospholipids on

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Abbreviations: PLC, phosholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdEtOH, phosphatidylethanol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

potassium oxalate (1%)-impregnated silica gel H plates (Analtech, Newark, DE) by using the solvent system of chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, by vol). The choline and ethanolamine metabolites were fractionated on Dowex-50-W(H^+)-packed columns (Bio-Rad Econo columns, Richmond, CA; 1-mL bed volume) as described by Cook and Wakelham (26) with the modifications described previously (27). The metabolites of [^{14}C]-ethanolamine and [^{14}C]choline were further identified by thin-layer chromatography (28). Contamination of the [^{14}C]ethanolamine fraction by [^{14}C]choline was less than 1%.

RESULTS

Concentration-dependent effects of PLC on the formation of choline and choline phosphate. We have shown (28) that in NIH 3T3 fibroblasts, labeled with [^{14}C]choline until radioisotopic equilibrium was achieved (48 h), PMA-induced formation of [^{14}C]choline from the prelabeled cellular PtdCho occurs by a PLD-mediated mechanism. In addition, we have demonstrated (23) that treatment of fibroblasts with PLC results in a significant increase in 1,2-diacylglycerol. As shown in Figure 1a, 0.25–1.5 U/mL concentrations of *B. cereus* PLC enhanced the formation of [^{14}C]choline from [^{14}C]PtdCho in a concentration-dependent manner. At an optimal stimulatory concentration of PMA (100 nM), the stimulatory effects of PLC were nonadditive with that of PMA, suggesting that these agents were acting through the same mechanism (Fig. 1a).

NIH 3T3 fibroblasts contain the α-, δ-, ε- and ζ-PKC isozymes. Treatment of fibroblasts with 300 nM PMA for 24 h almost completely down-regulates PKC-α (29), and also decreases the cellular levels of PKC-δ and PKC-ε by 75–90% (data not shown). In contrast, prolonged (24 h) PMA-treatment had no effect on the cellular level of PKC-ζ (N. Garamszegi and Z. Kiss, unpublished data). Prolonged (24 h) treatment of fibroblasts with PMA (300 nM) significantly enhanced the formation of [^{14}C]choline from labeled PtdCho, indicating partial activation of PLD (Fig. 1a). After chronic (24 h) treatment with PMA, neither newly added PMA (not shown) nor PLC (Fig. 1a) had any further effect on PtdCho hydrolysis.

Treatment of fibroblasts with PMA for 20 min or 24 h caused only little, if any, changes in PLC-catalyzed formation of choline phosphate (Fig. 1b). Thus, the inability of PLC to enhance [^{14}C]choline formation in the presence of PMA was not due to PMA-induced inactivation of PLC.

In the presence of ethanol, activated PLD catalyzes the formation of the metabolically more stable PtdEtOH. As shown in Figure 2, 0.75–1.5 U/mL concentrations of exogenous PLC greatly enhanced the formation of PtdEtOH in the presence of 200 mM ethanol. In agreement with the data in Figure 1, PLC failed to enhance PtdEtOH synthesis in the presence of PMA (Fig. 2).

In NIH 3T3 fibroblasts, activated PLD was shown to hydrolyze PtdEtn in addition to PtdCho (24,25,28–30). Thus, it was of interest to examine possible stimulation of PtdEtn hydrolysis by exogenous PLC. For this study, suspended [^{14}C]ethanolamine-labeled fibroblasts were used, because they were found to contain much lower background levels of unincorporated [^{14}C]ethanolamine compared to attached fibroblasts (24,27). Both in [^{14}C]-choline-labeled attached fibroblasts (Fig. 3a) and [^{14}C]-ethanolamine-labeled suspended fibroblasts (Fig. 3b), 0.75–1.5 U/mL concentrations of PLC rapidly, and to a similar extent, stimulated PLD-mediated hydrolysis of the respective labeled phospholipids. However, the stimula-

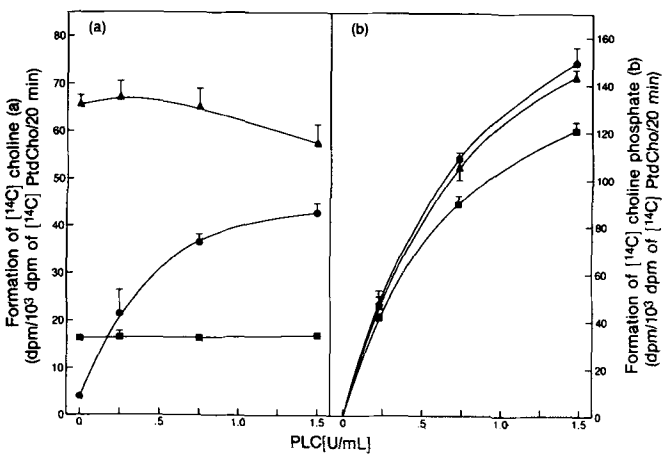

FIG. 1. Concentration-dependent effects of phospholipase C (PLC) on phosphatidylcholine (PtdCho) hydrolysis in NIH 3T3 fibroblasts. Fibroblasts were labeled with [^{14}C]choline for 48 h, followed by incubation of labeled fibroblasts for 20 min in the absence (●) or presence of 100 nM phorbol 12-myristate 13-acetate (PMA) (▲). In one set of experiments (■), cells were treated with 300 nM PMA for the last 24 h of the labeling period. [^{14}C]Choline (a) and [^{14}C]choline phosphate (b) were separated by ion-exchange chromatography. The ^{14}C content of PtdCho was 883000 and 819000 dpm/10^6 cells in the untreated and PMA-pretreated fibroblasts, respectively. Each point represents the mean ± SE of three incubations. Similar results were obtained in two other experiments.

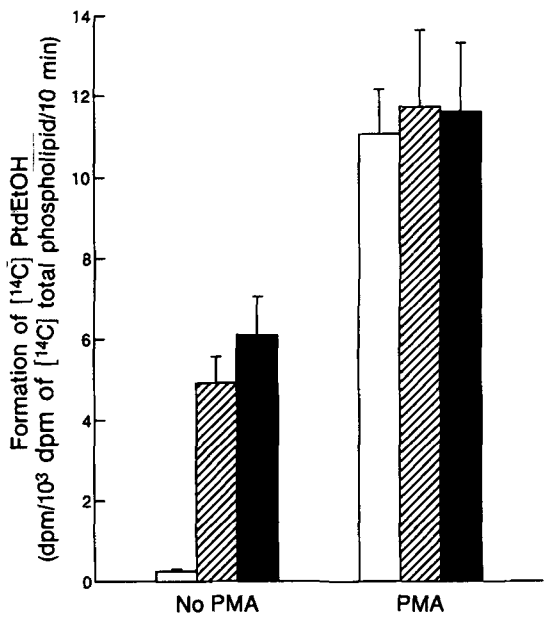

FIG. 2. Stimulatory effects of PLC and PMA on the formation of phosphatidylethanol (PtdEtOH) in [^{14}C]palmitate-labeled NIH 3T3 fibroblasts. Suspended [^{14}C]palmitate-labeled NIH 3T3 fibroblasts were incubated in the presence of 200 mM ethanol for 10 min. PLC was absent (open bar) or present at a concentration of 0.75 U/mL (hatched bar) or 1.5 U/mL (closed bar). When present, the concentration of PMA was 100 nM. Data are the mean ± SE of four incubations. Similar results were obtained in two other experiments. Abbreviations as in Figure 1.

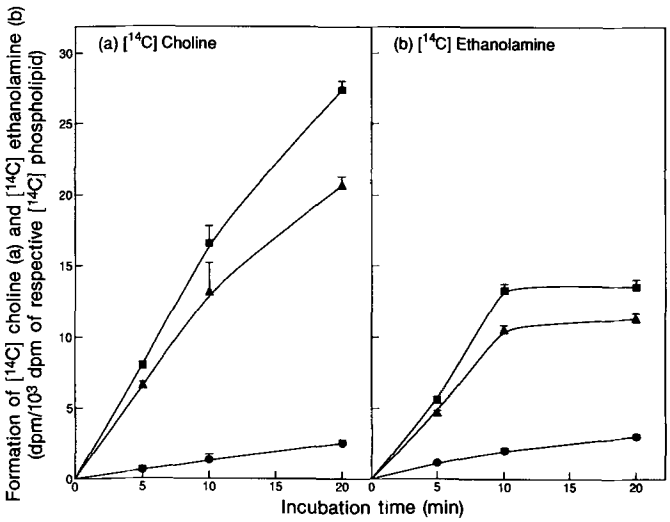

FIG. 3. Comparison of the time-dependent effects of PLC on the hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts. Fibroblasts were labeled with [^{14}C]choline (a) or [^{14}C]ethanolamine (b) for 48 h. Attached [^{14}C]choline-labeled and suspended [^{14}C]ethanolamine-labeled fibroblasts were incubated for 5–20 min in the absence (●) or presence of 0.75 U/mL of PLC (▲), or 1.5 U/mL of PLC (■). The [^{14}C]content of PtdCho and PtdEtn was 0.966×10^6 and 1.29×10^6 dpm/10^6 cells, respectively. Each point represents the mean ± SE of three incubations. Similar results were obtained in three other experiments. Abbreviations as in Figure 1.

tory effect of PLC on PtdEtn hydrolysis appeared to be rather transient (lasting only for 10 min) compared to PLC-induced PtdCho hydrolysis. One possible, and the most likely, explanation for this difference is that scraping decreased the sensitivity of fibroblasts to PLC. In support of this possibility, the stimulatory effects of PLC on PtdCho hydrolysis in [^{14}C]choline-labeled suspended fibroblasts also lasted for only 10–15 min (data not shown).

In NIH 3T3 fibroblasts, the methylation process is a relatively minor pathway. Thus, after labeling of fibroblasts with [^{14}C]ethanolamine for 48 h, only 3% of total phospholipid-associated ^{14}C-activity was present in PtdCho (data not shown). This, coupled with efficient separation of ^{14}C-labeled metabolites, ensured that contamination of the [^{14}C]ethanolamine fraction by [^{14}C]choline was minimal (less than 1%).

DISCUSSION

We have shown that treatment of NIH 3T3 fibroblasts with exogenous PLC leads to PKC-dependent activation of PLD. This indicates that 1,2-diacylglycerol, generated through the action of exogenous PLC, can stimulate not only the phosphorylating activity of PKC (20), but it can also enhance the ability of PKC to activate PLD by a non-phosphorylating mechanism (21). Because activated PLD generates the potent mitogen phosphatidic acid (13–19), this phospholipid product could be involved, at least in principle, in the mediation of mitogenic effects of PLC.

While chronic treatment of cells with PMA completely abolished the stimulatory effect of PLC on PLD activity (this work), such treatment failed to prevent the mitogenic effect of PLC (1). Therefore, a direct role of phosphatidic acid in the mediation of mitogenic effect of PLC is unlikely. However, in view of the present finding that prolonged treatment of fibroblasts with PMA results in permanent partial activation of PLD, the possibility that increased formation of phosphatidic acid by PLD is a prerequisite for the mitogenic action of PLC cannot be excluded. Further experiments are required to distinguish between these possibilities.

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Effect of Clofibric Acid on the Turnover of the Fatty Acid-Binding Protein Identified in Cultured Endothelial Cells from Bovine Aorta

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Several types of fatty acid-binding proteins are found in mammalian cells. Cultured endothelial cells from bovine aorta were shown to contain exclusively the cardiac-type fatty acid-binding protein (cFABP) with a mean concentration of 90 ng cFABP/mg extract protein. Only small variations were observed from passage to passage. In pulse-chase labeling experiments with L-[^{35}S]methionine, a half-life of 4.0 d was measured for cFABP which is about two times longer than the average half-life of the extracted proteins. These data imply that in aortic endothelial cells cFABP is not subject to short-term regulation. However, addition of clofibric acid to the culture medium led to a shortening of the half-life of cFABP, which was compensated for by an increase in its biosynthesis. The turnover of the bulk of extract proteins remained unchanged when the cells were challenged with clofibric acid.
Lipids 28, 483–486 (1993).

The fatty acid-binding proteins of the hepatic (hFABP), intestinal (iFABP) and cardiac (cFABP) type belong to a group of structurally well, but functionally ill-defined 14–15 kDa proteins that are variably expressed in mammalian cells. To date, hFABP expression appears to be limited to liver and intestinal cells, iFABP expression to the epithelial cells that line the intestinal tract, whereas cFABP is expressed in a broad range of tissues including heart, skeletal muscle, kidney, brain, stomach and placenta (1). Immunoblotting techniques and protein A-gold labeling revealed a complex distribution pattern for cFABP in the heart. The protein was detected in the cytosol of heart muscle cells where it associates with myofibrils, and in the mitochondrial matrix and in the nucleus (2,3). It is also present in heart capillary endothelium (3), in cultured endothelial cells of humans and rats (4) and in rat aortic tissue where its expression is specifically suppressed within 3 wk by experimentally induced hypertension (5). The cFABP contents of rat heart as well as the hFABP contents of rat liver are augmented by long-term feeding of high-fat diets (6). In response to clofibrate and other lipid-lowering drugs, however, the cFABP level in rat heart is not affected (7) which is in contrast to the elevated levels observed for hFABP in liver and intestinal cells (1). The data suggest that FABP are not rapidly regulated and, indeed, the half-life of hFABP in rat liver was found to be 3.1 d (8), and the half-life of cFABP in cultured neonatal cardiomyocytes was 2.5 d (9).

The occurrence of FABP types in cells of the vascular system and the strategic position of the endothelium at the interface between blood and tissue argue for a transporter function of this protein for fatty acids. With this in mind, we investigated the regulation of FABP in cultured endothelial cells from bovine aorta. We report here data on the half-life of FABP of the cardiac type and on the turnover of cFABP in endothelial cells that were treated with clofibric acid.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; cFABP, hFABP, iFABP, cardiac, hepatic and intestinal fatty acid-binding protein, respectively; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

MATERIALS AND METHODS

Materials. L-[^{35}S]Methionine (1100 Ci/mmole) was purchased from Amersham-Buchler (Braunschweig, Germany); Eagle's minimum essential medium and Hanks' salt solution were from Seromed (Munich, Germany). Penicillin and streptomycin were from Boehringer (Mannheim, Germany), protein A-Sepharose and clofibric acid (2-(*p*-chlorophenoxy)-2-methyl-propionic acid) from Sigma (Deisenhofen, Germany) and nitrocellulose paper (0.1 μm) from Schleicher and Schuell (Dasseln, Germany).

Cell cultures, conditions of preincubation and protein labeling. Endothelial cells were derived from bovine aorta, cultured and passaged in Eagle's minimum essential medium supplemented with 10% of fetal calf serum (Conco, Wiesbaden, Germany) in 25-cm^2 Nunc plastic flasks as outlined earlier (10). After cultures reached confluency, the medium was removed and cells were washed two times with 2 mL Hanks' salt solution. Preincubation with methionine-free Waymouth's MAB 87/3 medium (Gibco, Eggenstein, Germany) and labeling with L-[^{35}S]methionine were carried out in a manner analogous to that described (11). Per 25-cm^2 flask, 100–150 μCi were applied in a total volume of 1.9 mL. The pulse was terminated after 5 h either by harvesting or by feeding the cultures with complete tissue culture medium. Clofibric acid was solubilized in Eagle's minimum essential medium, and preincubation was carried out as described in the legends to Tables 1 and 2.

Immunoprecipitation. At the end of the incubation time, the cell layer was washed two times with 2 mL Hanks' salt solution and extracted with 0.4 mL detergent buffer [0.5% (wt/vol) sodium deoxycholate, 0.5% (vol/vol) Triton X-100, 1 M NaCl and proteinase inhibitors in 0.1 M Tris-HCl (pH 7.4)] for 90 min at room temperature and centrifuged (10 min, 10,000 × *g*). An aliquot of the resulting supernatant was treated with 12.5% (wt/vol) trichloroacetic acid (TCA) overnight at 4°C, the precipitate was washed twice with 10% (wt/vol) TCA and finally dissolved in 0.2 mL of 2.5 M NaOH ("extract proteins"). After neutralization, the radioactivity incorporated into extract proteins was measured by liquid scintillation counting. The remaining supernatant was shaken end over end with preimmune IgG-coated protein A-Sepharose (3 mg/25-cm^2 flask) at 4°C for 12–24 h. This step was repeated five times to remove unspecifically bound proteins. Sepharose was spun down, and the supernatant was transferred to a tube containing the same amount of protein A-Sepharose coated with affinity purified anti-cFABP-IgG or anti-Cathepsin D-IgG, respectively. After shaking for 20–24 h, the mixture was washed three times with 1 mL detergent buffer (see above) and two times with 1 mL of 10 mM sodium phosphate (pH 7.4) containing 0.15 M NaCl. The pellet was solubilized in sodium dodecyl sulfate

(SDS) sample buffer as described (11). Sepharose was removed by centrifugation and the immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Other methods. SDS-PAGE was performed in slab gels (12.5 cm long and 1.5 mm thick, separating gel 15% and stacking gel 5.85% acrylamide) (12), and the gels were then prepared for fluorography (13). For quantitative determinations, appropriate gel areas were cut out, swollen in water and treated with dimethyl sulfoxide to remove 2,5-diphenyloxazole. There was no loss in radioactivity during these procedures. Before determination of radioactivity, samples were dissolved in 2.2 M *ortho*-periodic acid for 5–10 min at 80–90°C, and after cooling the radioactivity was counted in 10 mL Instagel (Packard, Frankfurt, Germany). Antibodies against various FABP types were raised, purified and used in Western blots in a manner outlined earlier (2). cFABP was quantitated with the aid of a specific enzyme-linked immunosorbent assay (ELISA) (2) and protein by the bicinchoninic acid assay (14) with ovalbumin as standard.

Statistical analysis. Data are presented as means ± SD of n determinations. The significance of differences was evaluated by Student's *t*-test.

RESULTS

Characterization and quantitation of FABP. With all types of FABP known to occur in bovine tissue as well as respective antibodies on hand, SDS-PAGE and Western immunoblotting defined the FABP present in the extract of cultured bovine aortic cells as a cardiac-type protein (Fig. 1). The sensitivity of the sandwich-ELISA employed for quantitation was in the range of 0.05 to 1.0 ng cFABP per mL and allowed the determination of the minute amounts present in the extract. The content was ~90 ng cFABP/mg extract protein with variations of up to ±20% being observed from passage to passage.

Determination of cFABP half-life. Endothelial cells were pulse-labeled for 5 h with [^{35}S]methionine and chased for up to 12 d. [^{35}S]Methionine could be used as radioactive precursor, because bovine cFABP contains three methionine residues (15,16). The immunoprecipitation of [^{35}S]cFABP was of sufficient quality (Fig. 2) and was quantitative, as less than 5% of the total cFABP were found by the ELISA technique in the supernatant after immunoprecipitation. The band appearing around 30 kDa on gels after SDS-PAGE could only be detected after immunoprecipitation, but not in Western blots and it could be quenched by unlabeled cFABP. The radioactivity found in this band was about 68% of that in cFABP. Small molecular mass bands indicate the gel front. For the determination of cFABP half-life a log cpm *vs.* time plot was constructed which yielded straight lines with correlation coefficients from 0.97 to 0.99 (Fig. 3). From the decay curve, a half-life of 4.0 d was calculated for cFABP (15 kDa protein) and of 1.8 d for total extract proteins. Interestingly, the ~30 kDa cross-reactive protein also exhibited a half-life of 4.0 d. The sequence of the bovine cDNA for cFABP (15) excludes the possibility of the expression of a higher molecular mass precursor of cFABP and hence, the nature of this cross-reacting protein remains to be determined.

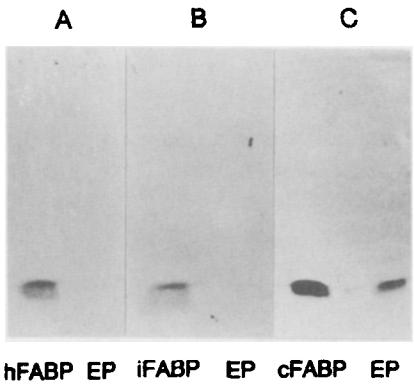

FIG. 1. Determination of fatty acid-binding protein (FABP) type expressed in aortic endothelial cells. Western blots after 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 20 μg extract proteins (EP) and of 100 ng FABP, each. Immunostaining was carried out with anti-hepatic (h)FABP antibodies (A), anti-intestinal (i)FABP antibodies (B), and anti-cardiac (c)FABP antibodies (C).

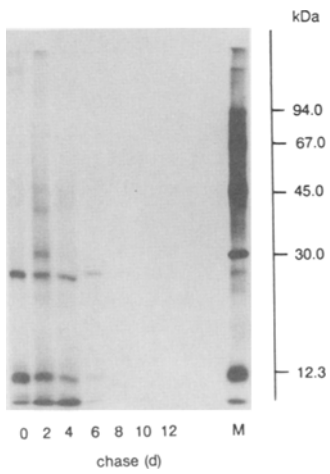

FIG. 2. Determination of cardiac fatty acid-binding protein half-life. After a 5-h pulse with 120 μCi of [^{35}S]methionine, cultures were chased for the times indicated. Autoradiogram after 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated proteins; M, marker proteins (phosphorylase b, bovine serum albumin; ovalbumin; carbonic anhydrase; cytochrome c, migrates as 14–15 kDa protein).

Influence of clofibric acid on cFABP metabolism. Clofibric acid was admixed to the culture medium in the concentrations shown in Tables 1 and 2. After administration for 3 to 5 d (order of cFABP half-life), the concentrations of cFABP and of extract proteins were determined. Compared with untreated controls, the values remained constant within the margin of error (Table 1). At a concentration of 5 mM clofibric acid, toxic effects were observed as some of the endothelial cells became detached from the dishes and, therefore, only concentrations up to 1 mM were used in subsequent experiments. Interestingly, clofibric acid did influence the *de novo* synthesis of cFABP. After preincubation of the cells for 2 d with this drug and a subsequent 5-h pulse with [^{35}S]methionine, a dose-dependent increase of up to 50% of the radioactivity

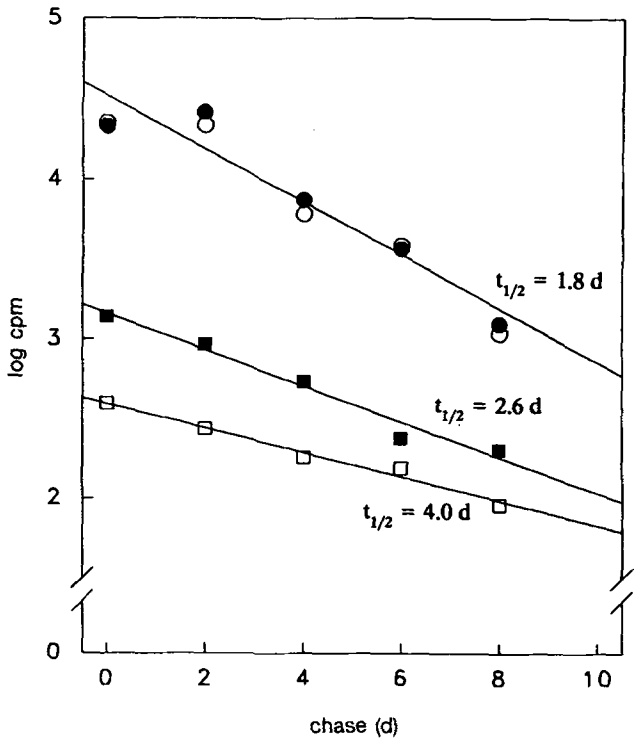

FIG. 3. Decay of radioactivity in cardiac fatty acid-binding protein (cFABP) and extract proteins following pulse labeling with [^{35}S]methionine in the presence or absence of clofibric acid. From experiments as shown in Figure 2, bands with cFABP (15 kDa protein) were excised from the gel and subjected to scintillation counting. Radioactivity in extract proteins was determined after precipitation with trichloroacetic acid. ●, Extract proteins + clofibric acid; ○, extract proteins − clofibric acid; ■, cFABP + clofibric acid; □, cFABP − clofibric acid.

TABLE 1

Effect of Clofibric Acid on the Concentration of cFABP[a]

Clofibric acid	cFABP	Extract proteins
mM	ng/mg protein (n = 18)	μg/culture flask (n = 18)
0.0	90 ± 20	268 ± 22
0.2	100 ± 20	265 ± 40
1.0	80 ± 40	261 ± 62
5.0	70 ± 40	221 ± 49

[a]Prior to harvesting, endothelial cell cultures were treated with the drug for 3–5 d. Proteins were determined as described under Materials and Methods. Values represent means ± SD of n determinations. cFABP, cardiac fatty acid-binding protein.

incorporated into cFABP was observed as compared to untreated controls (Table 2). This increase was detected in four independent experiments whereas the incorporation of the radioactivity into extract proteins was not significantly affected by clofibric acid. Moreover, in additional immunoprecipitation experiments with treated (1 mM clofibric acid) and untreated cells we determined the half-life of cathepsin D, a lysosomal protein structurally and functionally unrelated to cFABP, and could not find any difference (data not shown). Taking all controls together, we concluded that clofibric acid did not affect the [^{35}S]-methionine pool, and consequently we studied the cFABP turnover in the presence of 1 mM clofibric acid analogously as described above, except that the chase was shortened to 8 d. From the decay curves (Fig. 3), a half-life of 2.6 d was calculated for cFABP and of 1.8 d for extract proteins. The shorter half-life corresponded to the increase in cFABP synthesis since cellular cFABP levels were not altered. On the other hand, clofibric acid had no effect on the half-life of extract proteins or cathepsin D (see above).

TABLE 2

Effect of Clofibric Acid on the *de novo* Synthesis of cFABP[a]

Clofibric acid	Radioactivity in cFABP	Radioactivity in extract proteins
mM	(%) n = 4	(%) n = 4
0.0	100 ± 10	100 ± 11
0.2	129 ± 17[b]	97 ± 17
1.0	152 ± 17[c,d]	79 ± 26

[a]Endothelial cell cultures were pretreated with the drug for 2 d and then pulsed with 120 μCi [^{35}S]methionine. After 5 h, cells were lysed and proteins analyzed as described under Materials and Methods. Values represent means ± SD of n determinations. cFABP, cardiac fatty acid-binding protein.
[b]Significantly different ($P < 0.025$) from untreated cells.
[c]Significantly different ($P < 0.0025$) from untreated cells.
[d]Significantly different ($P < 0.05$) from cells treated with 0.2 mM clofibric acid.

DISCUSSION

Aortic endothelial cells, as shown here, and heart endothelium (3,4) express FABP of the cardiac type. The identifications are based on SDS-PAGE and immunological cross-reactivity, the latter requiring at least a 70–80% homology in primary structures of FABP when polyclonal antibodies are used (17). The cross-reacting endothelial protein with ~30 kDa may be related to cFABP; however, it was found only after biosynthetic labeling and not in Western blots of cell extracts. This suggests either a high [^{35}S]methionine content of the cross-reacting species or the formation of a dimer of newly synthesized cFABP. Previously the existence of a disulfide-linked dimer of cFABP has been shown, whose formation was facilitated under denaturing conditions due to the exposure of the protein's single cysteine residue (18). Such dimerization seems to be unlikely because the immunoprecipitate was treated with 10 mM dithiothreitol in SDS sample buffer for 6 min at 95°C but cannot be excluded.

The concentration of cFABP in aortic endothelial cells is 90 ± 20 ng/mg extract protein and stays within this range from passage to passage (data not shown). It is much smaller than that of cFABP in the capillary endothelium of rat heart muscle as calculated by the protein A-gold method (3) as well as the 3.18 ± 0.651 μg/mg protein of bovine heart cytosol determined by the cFABP-specific ELISA (2). The small amount of cFABP in the aortic endothelial cells could be due to the fact that in cultured cells the expression is lower than *in vivo*. It could also reflect the different functions of heart aorta and heart muscle. The half-life of 4.0 d for cFABP was calculated from a representative experiment, variations were from

3.6 to 4.6 d possibly due to the biological variability in different cell strains. These half-lives are longer than the 3.1 d determined for hFABP half-life in rat liver (8) and the 2.5 d found for cFABP half-life in cultured neonatal cardiomyocytes (9). Although the functional role(s) of cFABP in endothelial cells remain(s) to be defined, the data elaborated here imply that cFABP plays a housekeeping role and is not subject to short-term regulation. It may thus sustain the intracellular flux of fatty acids under fast changes of nutritional states and may provide a pool of fatty acids available for membrane biosynthesis and production of metabolites of the arachidonic acid cascade.

For the studies on the effect of clofibrate, clofibric acid was used instead of its ethyl ester, because of its better solubility in the culture medium. The drug did not affect the concentration of cFABP in endothelial cells. Our data are consistent with the observation that hypolipidemic drugs of this type do not enhance the cFABP content in rat heart (7). The concentration of hFABP in liver, on the other hand, is increased by clofibrate proportional to the effect of this drug on peroxisomal proliferation without being necessarily linked to the latter (19,20). The mechanism by which hypolipidemic drugs influence the concentration of hFABP is still unknown. Our findings reveal an increased *de novo* synthesis of cFABP in endothelial cells following clofibric acid treatment, while the cFABP content remains unchanged. In contrast to Bass *et al.* (21), who have found that the hFABP concentration was increased in clofibrate-treated rats without changes in the turnover, cFABP turnover was accelerated in endothelial cells after clofibric acid treatment. These observations suggest different roles of liver and heart FABP types in cellular lipid metabolism. Their specific tissue distribution is another important indication in this respect (22). The fact that in clofibrate-treated rats the hFABP mRNA concentration is increased in parallel with the hFABP concentration indicates a pretranslational regulation of hFABP (21). The markedly reduced expression of cFABP mRNA in aorta during hypertension, concomitant with a reduction of immunologically detectable protein, suggests transcriptional regulation of cFABP synthesis (5). In further studies the isolation of total cellular RNA and Northern blot analysis may give an insight into cFABP mRNA concentration and its response to clofibric acid treatment of endothelial cells.

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Transformation of Fatty Acid Hydroperoxides by Alkali and Characterization of Products

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It has previously been determined that (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid was mainly converted into (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid by 5 N KOH with preservation of the stereochemistry of the reactant [Simpson, T.D., and Gardner, H.W. (1993) *Lipids 28,* 325–330]. In addition, about 20–25% of the reactant was converted into several unknown by-products. In the present work it was confirmed that the stereochemistry was conserved during the hydroperoxy-diene to hydroxy-diene transformation, but also, novel by-products were identified. It was found that after only 40 min reaction (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid accumulated to as much as 7% of the total. Later, (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid began to disappear, and several other compounds continued to increase in yield. Two of these compounds, 2-butyl-3,5-tetradecadienedioic acid and 2-butyl-4-hydroxy-5-tetradecenedioic acid, were shown to originate from (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid, and they accumulated up to 2–3% each after 4 to 6 h. Some other lesser products included 11-hydroxy-9,12-heptadecadienoic acid, 3-hydroxy-4-tridecenedioic acid, 13-oxo-9,11-octadecadienoic acid and 12,13-epoxy-11-hydroxy-9-octadecenoic acid. Except for the latter two, most or all of the compounds could have originated from Favorskii rearrangement of the early product, (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid, through a cyclopropanone intermediate.
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It is known that organic hydroperoxides are converted into the corresponding hydroxy compounds by alkali (for a review, see Ref. 1). As a result of saponification of autoxidized fatty acid methyl esters, such a conversion of fatty ester hydroperoxide to fatty acid hydroxide was first reported in 1961 (2). A number of other "nucleophiles" also converted linoleic acid hydroperoxide into the corresponding hydroxy fatty acid (3), but the earlier reports included few details on the mechanism or the structure of the product(s). Recently, the kinetics of the KOH-catalyzed transformation of (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPOD) and (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13*S*-HPOT) have been reported, including the observation that the stereoconfiguration of the reactant hydroperoxides is preserved in the product hydroxy fatty acid (4).

In this communication we not only confirm that the alkali-promoted hydroperoxide-to-hydroxide transformation proceeds without change of the stereoconfiguration, but also describe the isolation and structure determination of several novel oxylipins which are formed from fatty acid hydroperoxides in the presence of alkali. At least two of the novel compounds appear to be derived from an intermediate fatty acid, (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid.

MATERIALS AND METHODS

13S-HPOD preparation. [1-^{14}C]- and [U-^{14}C]linoleic acids (Amersham, United Kingdom) were diluted with unlabeled linoleic acid (Nu-Chek-Prep, Elysian, MN) and purified by silicic acid chromatography. Labeled and unlabeled 13*S*-HPOD (chemical and radiochemical purity in excess of 95%) were prepared from the linoleic acid samples as described previously (5). For time-course experiments [1-^{14}C]-labeled 13*S*-HPOD was utilized (specific radioact., 1.9 kBq/μmol); [U-^{14}C]-labeled 13*S*-HPOD (specific radioactivity, 0.2 kBq/μmol) was used for product isolation.

Reactant conditions. ^{14}C-labeled 13*S*-HPOD (2.3 mM) was reacted in 5 N KOH (Elektrokemiska Aktiebolaget, Bohus, Sweden) at 35°C with periodic vigorous agitation. Products were recovered by rapid neutralization to pH 4.0 with 1 M oxalic acid (about 3 vol), and the fatty acids were immediately partitioned into $CHCl_3$ by addition of 3 vol of $CHCl_3/CH_3OH$ (2:1, vol/vol) based on the volume of the neutralized reaction mixture. The $CHCl_3$ layer was washed twice with H_2O, and the recovered product mixture was esterified with CH_2N_2 in diethyl ether/CH_3OH (9:1, vol/vol).

Chromatographic methods. The esterified products were preparatively separated into fractions by low-pressure liquid chromatography (LPLC). In a typical isolation, 98 mg of product was applied to a column (1.5 cm i.d.) packed with 9 g SilicAR CC4 (Mallinckrodt, Paris, KY) in hexane. Elution was sequentially done with 150 mL each of 3.5, 7.5 and 15% diethyl ether in hexane followed by 100 mL of diethyl ether. The eluent was collected in 30-mL fractions. The first 210 mL contained methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate, dimethyl 2-butyl-3,5-tetradecadienedioate, and some 13*S*-HPOD (methyl ester), and the fraction collected between 210 and 300 mL consisted mainly of 13*S*-HPOD (methyl ester) and small amounts of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate and (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid (13*S*-HOD) as its methyl ester. Material eluting between 300 and 390 mL was comprised of 13*S*-HOD (methyl ester). Some 13*S*-HOD (methyl ester), other fatty ester dienols and dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate were obtained in the final elution between 390 and 540 mL.

Thin-layer chromatography (TLC) was done on precoated plates (Kieselgel 60, 0.25 mm thickness) obtained

[1]This research was completed while the corresponding author was on sabbatical leave at the Karolinska Institutet, Stockholm, Sweden.
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Abbreviations: GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; 13*S*-HOD, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid; 13*S*-HOT, (13*S*,9*Z*,11*E*,15*Z*)-13-hydroxy-9,11,15-octadecatrienoic acid; 13*S*-HPOD, (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid; 13*S*-HPOT, (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; IR, infrared; LPLC, low-pressure liquid chromatography; MC, (−)-menthoxycarbonyl derivative; NMR, nuclear magnetic resonance; OTMS (or TMSO), trimethylsilyloxy derivative; R_f, migration relative to the solvent front; TLC, thin-layer chromatography; TMS, trimethylsilyl cation; UV, ultraviolet.

from E. Merck (Darmstadt, Germany). Solvent A, comprised of hexane/ethyl acetate (4:1, vol/vol), was used for general separation of samples. Solvents B, C and D, hexane/ethyl acetate (85:15, vol/vol 93:7, vol/vol and 7:3, vol/vol, respectively), were utilized for additional purification of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate (Solvent B, $R_f = 0.51$), dimethyl 2-butyl-3,5-tetradecadienedioate (Solvent C, $R_f = 0.33$), and dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate (Solvent D, $R_f = 0.51$). Other TLC separations employed hexane/ethyl acetate (3:1, vol/vol, Solvent E), hexane/diethyl ether (3:2, vol/vol, Solvent F), hexane/acetone (7:1, vol/vol, Solvent G). Separated components were localized by spraying with aqueous 0.1% 8-anilino-1-naphthalenesulfonic acid (Na salt) from Aldrich Chemical (Milwaukee, WI) and viewing under ultraviolet (UV) light. Radioactivity on TLC plates was measured by a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh SE/30 PC.

Spectral and instrumental analyses. Gas chromatography/mass spectrometry (GC/MS) was done with a Hewlett-Packard Model 5970B mass selective detector connected to a Hewlett-Packard Model 5890 gas chromatograph (Avondale, PA) equipped with a methyl silicone capillary column (length, 11 m; film thickness, 0.33 μm). Usually, the temperature was increased from 120 to 200°C at 10°C/min and then held isothermally at 200°C. For the derivative obtained by reaction of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate with $LiAlH_4$ followed with treatment by menthoxycarbonyl chloride, oxidative ozonolysis and methyl ester formation, the temperature was increased to 240°C and held.

Gas-liquid chromatography (GLC) of (−)-menthoxycarbonyl (MC) derivatives was done with a Hewlett-Packard Model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm); separation of methyl MC-2-hydroxyheptanoates required that the temperature was increased from 190 to 260°C at 2°C/min (*S*- and *R*-2-hydroxy-isomers eluted at 10.38 and 10.51 min, respectively). Dimethyl MC-malate derived from (13*S*,9*Z*,11*E*,15*Z*)-13-hydroxy-9,11,15-octadecatrienoic acid (13*S*-HOT) was separated by a DB-210 capillary column (length 15 m; film thickness, 0.25 μm; J & W Scientific, Folsom, CA) by temperature programming from 160 to 210°C at 2°C/min (*S*- and *R*-malate isomers eluted at 11.72 and 12.15 min, respectively). Infrared (IR) spectra were recorded with a Perkin-Elmer Model 257 IR spectrophotometer (Norwalk, CT), and UV spectra were obtained on a Hewlett-Packard Model 8450A UV/VIS spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Model WM-300 spectrometer (Karlsruhe, Germany) with samples dissolved in C^2HCl_3. Radioactivity was measured with a Packard Tricarb Model 4450 liquid scintillation counter (Downers Grove, IL).

Chemical methods. The stereochemistry of hydroxydiene and hydroxytriene fatty esters was determined by GLC separation of MC derivatives obtained after oxidative ozonolysis (6) using a recently modified procedure for analysis of dienols (7). Hydrogenation of samples dissolved in CH_3OH was completed under H_2 for 1 h with 5% Pd on $CaCO_3$.

Trimethylsilyloxy (OTMS) derivatives were prepared with trimethylchlorosilane/hexamethyldisilazane/pyridine (3:2:2, by vol). Methyl esters (0.5 mg) were transesterified with 50 μL of 0.17 M Na ethoxide overnight at 23°C, and the ethyl esters were recovered by $CHCl_3$ extraction after acidification. Epoxides (0.5 mg) dissolved in 1.5 mL H_2O/1,2-dimethoxyethane (2:1, vol/vol) were hydrolyzed by addition of 50 μL of 70% $HClO_4$ and incubation for 10 min at 23°C. Samples (1 mg or less) were reduced with 20 mg $LiAlH_4$ in 2 mL diethyl ether overnight at 23°C. Ketones (1 mg or less) were reduced with 5 mg NaB^2H_4 (Merck Sharp and Dohme, Montreal, Canada) in 1 mL CH_3OH for 30 min at 0°C.

RESULTS

Formation of the principal product, 13S-HOD. A 2.3 mM solution of ^{14}C-labeled 13*S*-HPOD in 5 N KOH was held at 35°C for several hours. Aliquots taken at various time points revealed that after acidification to pH 4, the ^{14}C-label was quantitatively extracted into $CHCl_3$ up to at least 2.5 h of reaction. Thereafter, there was a small decline of extractable label to 91% after 17 h of incubation.

As we previously reported (4), the principal product of the reaction was 13*S*-HOD. Although there were several minor products, 72–75% of the initial ^{14}C-label was recovered as 13*S*-HOD when the reaction was essentially complete after 4 h (Figs. 1 and 2). As seen in Figure 1, at least one of the minor products was transiently formed, and two others became more prominent later in the reaction.

We confirmed by spectral methods that the major product was 13*S*-HOD. The IR spectrum showed absorptions at 984 and 948 cm^{-1} consistent with the *E,Z*-configuration of a dienol (8). GC/MS of the OTMS-methyl ester revealed one major peak corresponding to the *E,Z*-dienol isomer containing a trace of the *E,E*-dienol. The main peak afforded a characteristic spectrum for this compound and localized the dienol moiety between C-9 and C-13 as follows: [*m/z* (percentage relative intensity, ion structure)] 382 (31, M^+); 367 (2, $[M - CH_3]^+$); 351 (2, $[M - CH_3O]^+$); 311 (59, $[M - CH_3(CH_2)_4]^+$; 225 (24, $[M - (CH_2)_7COOCH_3]^+$); 130 (58); 73 [100, trimethylsilyl cation (TMS)]. The 13-hydroxyl was localized by hydrogenation of 13*S*-HOD and GC/MS of the corresponding OTMS-methyl ester giving the following characteristic ions: 371 (3, $[M - CH_3]^+$); 355 (7, $[M - CH_3O]^+$); 339 (20, $[M - CH_3OH - CH_3]^+$); 315 (100, $[M - CH_3(CH_2)_4]^+$); 286 (18, $[M - CH_3(CH_2)_4CHO]^+$); 173 (90, $[M - (CH_2)_{11}COOCH_3]^+$); 73 (54, TMS).

It was previously shown by chiral-phase high-performance liquid chromatography that the stereoconfiguration of 13*S*-HPOD was conserved in the KOH reaction product, 13*S*-HOD (4). As shown in Table 1, we confirmed the previous result by an independent method employing GLC separation of MC derivatives (6,7). In addition, it was confirmed (Table 1) that 5 N KOH treatment of 13*S*-HPOT resulted in preservation of the stereoconfiguration of the corresponding product, 13*S*-HOT.

13*S*-HOD isolated from a reaction with KOH was reincubated with 5 N KOH for 4 h at 35°C, and the resulting material was examined by TLC as its methyl ester. One radio-labeled peak corresponding to 13*S*-HOD indicated that this compound is surprisingly stable to strong alkali treatment. Subsequent isolation of the methyl ester from

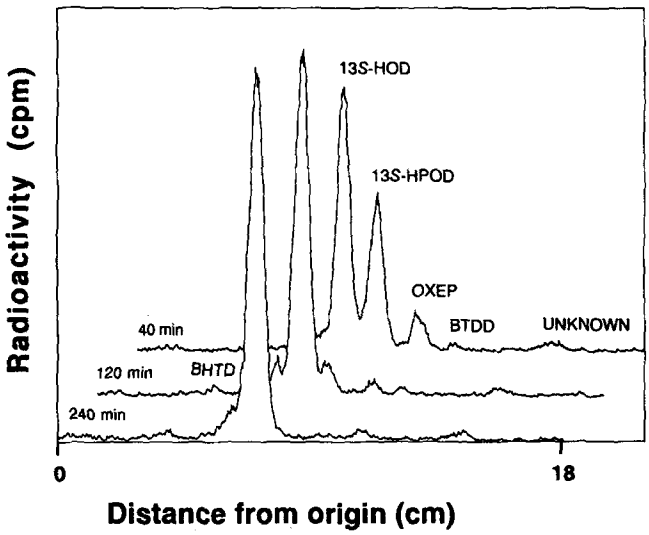

FIG. 1. Thin-layer chromatography (TLC) radiograms of products (as methyl esters) sampled at 40, 120 and 240 min from 5 N KOH treatment of 13*S*-HPOD at 35°C. TLC plates were developed with Solvent A, hexane/ethyl acetate (4:1, vol/vol). BHTD, 2-butyl-4-hydroxy-5-tetradecenedioic acid; BTDD, 2-butyl-3,5-tetradecadienedioic acid; 13*S*-HOD, (13*S*,9*Z*,11*E*)-13-hydroxy-9,11-octadecadienoic acid; 13*S*-HPOD, (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid; OXEP, (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid.

TLC and GC/MS of the OTMS ether confirmed that 13*S*-HOD was unchanged.

Other significant products. The time course of appearance of three of the most significant of the minor products, analyzed as their methyl esters, is plotted in Figure 2. The most transient of these was (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid which amounted to as much as 7% of the total products at its peak accumulation after 40 min. The two other products, 2-butyl-3,5-tetradecadienedioic acid and 2-butyl-4-hydroxy-5-tetradecenedioic acid, slowly accumulated for 4 h after which they reached a maximum of 2–3% of the total.

(9*Z*)-13-Oxo-*trans*-11,12-epoxy-9-octadecenoic acid, a novel product of 13*S*-HPOD, was isolated as its methyl ester by sequential LPLC and TLC. After a 40-min reaction of 13*S*-HPOD with 5 N KOH, a 6.6% yield was obtained (Table 2). Subsequently, the isolated methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate was subjected to spectral analyses. GC/MS afforded the mass spectrum shown in Figure 3, consistent with its proposed structure. An IR spectrum gave the following absorptions in wave number, intensity and assignment, respectively: 1743 cm^{-1}, strong, ester CO; 1714 cm^{-1}, medium, ketone CO; 880 cm^{-1}, weak, *trans*-epoxide; 835 cm^{-1}, weak, *cis*-epoxide. The ambiguity left in assigning the configuration of the epoxide based on the IR spectrum was clarified by the ^1H NMR spectrum which furnished a $J_{11,12}$ of 2.0 Hz, typical of *trans*-epoxides. Other ^1H NMR data are given as follows in chemical shifts, ppm and followed in parentheses by the number of protons, multiplicity, coupling constants and carbon assignments: 2.18 (2H, *m*, C-8); 5.79 (1H, *dt*, $J_{8,9} = 7.7$ Hz, $J_{9,10} = 10.9$ Hz, C-9); 5.03 (1H, *ddt*, $J_{9,10} = 10.9$ Hz, $J_{10,11} = 9.0$ Hz, $J_{8,10} = 1.4$ Hz, C-10); ~3.67 (partly obscured, apparent *dd*, C-11); 3.36 (1H, *d*,$J_{11,12} = 2.0$ Hz, C-12); 2.41 (2H, *m*, C-14); 3.66 (3H, *s*, ester methyl). The $J_{9,10} = 10.9$ Hz established that the 9,10-olefin had a *Z* configuration. The spin system was confirmed by correlative spectroscopy.

A number of derivatives of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate were examined by GC/MS. The most informative of these were from (i) solvolysis of the epoxide, (ii) hydrogenation, and (iii) hydrogenation followed by NaB^2H_4 reduction of the C-13 ketone. First, methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate was treated by $HClO_4/H_2O$ to solvolyze the allylic epoxide. Two components were observed upon GC/MS analysis of the silylated solvolysis product. The early eluting peak accounted for about one-third of the total ion intensity

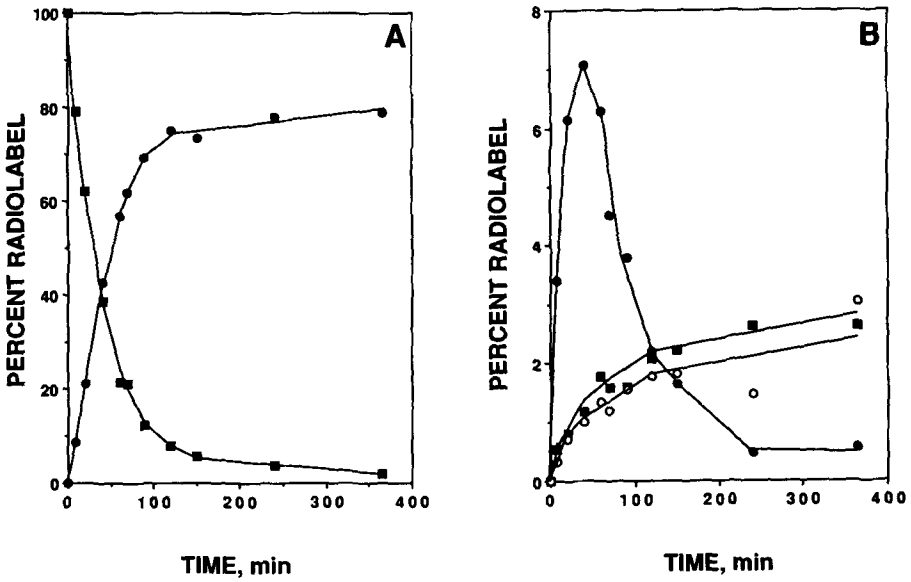

FIG. 2. Time course analysis of products from 5 N KOH treatment of 13*S*-HPOD at 35°C. TLC radiochromatograms of methyl esters were developed using Solvent A, and component peaks were integrated. Panel A: 13*S*-HPOD ■—■; 13*S*-HOD ●—●. Panel B: OXEP ●—●; BTDD ■—■; BHTD ○—○. Abbreviations as in legend of Figure 1.

TABLE 1

Chiral Analysis of 13-Hydroxy Fatty Acids Produced by KOH Treatment of 13-Hydroperoxides[a]

	Experiment 1 13-HOD[b]		Experiment 2 13-HOD[c]		Experiment 3 13-HOT[d]	
Sample	*S*	*R*	*S*	*R*	*S*	*R*
After KOH	97.8	2.2	98.4	1.6	98.7	1.3
Starting reactant	96.8	3.2	97.8	2.2	97.5	2.5
Remaining reactant	99.2	0.8	—	—	97.5	2.5

[a]The corresponding hydroxy fatty acids were isolated either after KOH treatment, after $NaBH_4$ reduction of starting hydroperoxide, or $NaBH_4$ reduction of hydroperoxide remaining after reaction with KOH.
[b]Samples were obtained as described in a previous study (4) reacting 2.3 mM (13*S*,9*Z*, 11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPOD) with 5 N KOH at 25°C for 90 min. 13-HOD, β-hydroxyoctadecadienoic acid.
[c]13*S*-HPOD (2.3 mM) was reacted with 5 N KOH at 35°C for 240 min.
[d]Samples were obtained as described previously (4) reacting 2.3 mM (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid with 5 N KOH for 50 min. 13-HOT, 13-hydroxy octadecatrienoic acid.

TABLE 2

Products Isolated from a Reaction of 13*S*-HPOD and 5 N KOH for Different Times[a]

	Percent composition at reaction time[c]	
Product[b]	40 min	4 h
13*S*-HOD	79	74
13*S*-HPOD		n.d.
(9*Z*)-13-Oxo-*trans*-11,12-epoxy-9-octadecenoic acid	6.6	n.d.
2-Butyl-3,5-tetradecadienedioic acid	0.6	2.0
2-Butyl-4-hydroxy-5-tetradecenedioic acid	0.8	2.0
2-Butyl-4-hydroxy-5-tetradecenedioic acid isomer[d]	0.1	0.5
3-Hydroxy-4-tridecenedioic acid[d]	n.d.[e]	0.4
C-10 to C-13 short-chain dioic acids[d]	n.d.[f]	1.6
13-Methoxy-9,11-octadecadienoic acid	0.5	0.7
13-Oxo-9,11-octadecadienoic acid[d]	0.1	0.2
11-Hydroxy-9,12-heptadecadienoic acid[d]	0.3	1.0
9-Hydroxy-10*E*,12*Z*-octadecadienoic acid[d]	0.8	1.0
9-Hydroxy-10*E*,12*E*-octadecadienoic acid[d]	0.1	trace
13-Hydroxy-9*E*,11*E*-octadecadienoic acid[d]	trace	trace
12,13-Epoxy-11-hydroxy-9-octadecenoic acid[d]	0.5	0.1
POLAR[d]	1.7	1.3
Unknown	1.5	1.6

[a]The 40-min and 4-h reactions employed 98 mg and 67 mg (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPOD), respectively.
[b]POLAR, polar compounds, including trihydroxyoctadecenoic acid, found near the origin of thin-layer chromatography (TLC) plates; unknown, nonpolar product found at TLC solvent front using Solvent A. (13*S*,9*Z*,11*E*)-13-Hydroxy-9,11-octadecadienoic acid (13*S*-HOD).
[c]Products (methyl esters) were quantitated by measuring radioactivity after separation by low-pressure liquid chromatography followed by TLC. Composition of mixtures was estimated by total ionization chromatograms using gas chromatography/mass spectrometry (GC/MS).
[d]Those fractions estimated by GC/MS total ionization chromatograms.
[e]n.d., not detected.
[f]Product may have been obscured by close migration of a major component.

and was consistent with methyl 13-oxo-11,12-*bis*(trimethylsilyloxy)-9-octadecenoate as follows: 455 (2, $[M - CH_3O]^+$); 387 (1.5, $[M - CH_3(CH_2)_4CO]^+$); 285 (77, $^+CHOTMSCH{=}CH(CH_2)_7COOCH_3$); 274 (100 TMS + $^+CHOTMSCO(CH_2)_4CH_3$); 259 (7, 274 − CH_3); 129 (24); 99 (7, $^+CO(CH_2)_4CH_3$); 73 (72, TMS). The later-eluting peak accounting for two-thirds of the total ion intensity had ions consistent with methyl 13-oxo-9,12-*bis*(trimethylsilyloxy)-10-octadecenoate as follows: 455 (4, $[M - CH_3O]^+$); 387 (95, $[M - CH_3(CH_2)_4CO]^+$); 300 (27, TMS + $CH_3(CH_2)_4COCHOTMSCH{=}CH^+$); 297 (64, 387-TMSOH); 259 (8); 99 (10, $^+CO(CH_2)_4CH_3$); 73 (100, TMS). As expected, solvolysis of the allylic epoxide gave products that could be explained by formation of the C-11 carbocation. Delocalization of the carbocation caused hydroxyl substitution at both C-11 and C-9. Next, hydro-

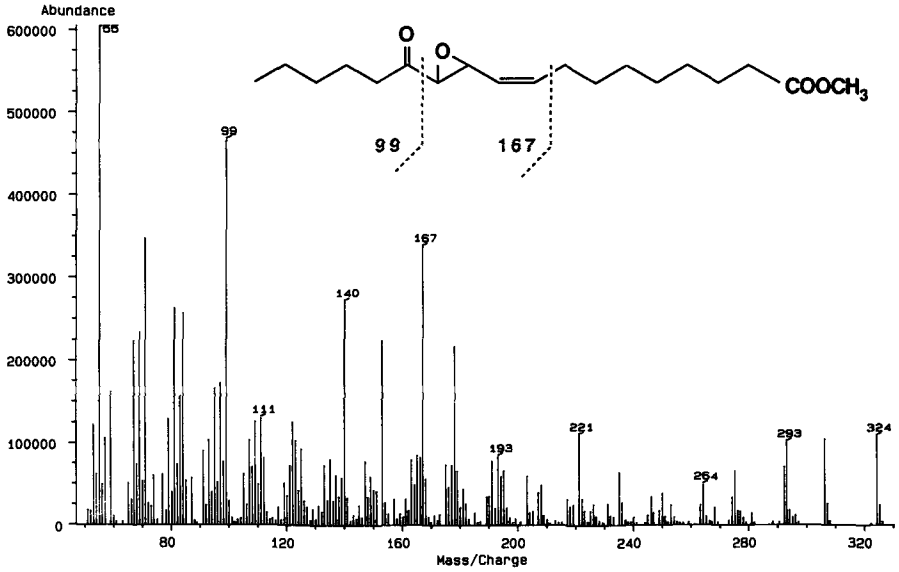

FIG. 3. Mass spectrum of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate.

genation of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate followed by OTMS derivatization afforded one GC/MS peak with ions indicative of methyl 13-oxo-12-trimethylsilyloxyoctadecanoate from hydrogenation of both the double bond and the allylic carbon at C-11 as follows: 385 (5, $[M - CH_3]^+$); 301 (100, $[M - CO(CH_2)_4CH_3]^+$); 73 (34, TMS). The C-13 ketone was localized by NaB^2H_4 reduction and OTMS derivatization of the hydrogenation product furnishing two separable isomers by GC/MS in approximately 3:2 ratio (presumably from *erythro* and *threo* diols). These two peaks gave essentially identical mass spectra; the first-eluting and largest of the two afforded the following ions: 444 (4, $[M - CH_3O]^+$); 374 (13); 301 (100, $^+CHOTMS(CH_2)_{10}COOCH_3$); 174 (51, $CH_3(CH_2)_4C^2HOTMS^+$); 73 (70, TMS). The *m/z* 174 ion showed that the ketone was located at C-13.

The (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid structure was also proven by synthesis. Reaction of 13*S*-HPOD with vanadium oxyacetylacetonate followed by methyl ester formation afforded *erythro* and *threo* isomers of methyl (9*Z*)-13-hydroxy-*trans*-11,12-epoxy-9-octadecenoate as previously described (9). Reduction of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate with $NaBH_4$ afforded two isomers that migrated at the same R_f with three different solvent systems (Solvents E–G) as the methyl esters of *erythro* and *threo* isomers synthesized by vanadium oxyacetylacetonate oxidation of 13*S*-HPOD. As estimated by spot intensity, $NaBH_4$ reduction of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate resulted in the formation of *erythro/threo* isomeric ratio of about 3:1. Oxidation by CrO_3 of the vanadium oxyacetylacetonate synthesized isomers resulted in the formation of methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate that was identical to the KOH-produced compound as shown by its GC/MS, TLC, IR spectrum and ^1H NMR spectrum.

2-Butyl-3,5-tetradecadienedioic acid, another novel product of 13*S*-HPOD, was produced by reaction of 13*S*-HPOD for 4 h with 5 N KOH, and this compound was isolated as its dimethyl ester in 2.0% yield by LPLC followed by TLC (Table 2). Dimethyl 2-butyl-3,5-tetradecadienedioate was characterized by its GC/MS, UV spectrum and GC/MS of its derivatives. The GC/MS (Fig. 4) showed the molecular ion at *m/z* 338, a strong $[M - CH_3OH]^+$ ion indicative of methyl ester(s), an *m/z* ion at 274 indicative of the loss of two CH_3OH molecules and two ions at *m/z* 279 and 278 indicative of a labile carboxylic ester function, $[M - 59]^+$ and $[M - 60]^+$. The absence of carbonyl and hydroxyl groups was demonstrated by lack of change in the GC/MS after treatment with $NaBH_4$ and silylating reagent. That this product was a dimethyl dicarboxylate was shown by transesterification with Na ethoxide followed by GC/MS of the product. The molecular ion increased as expected to *m/z* 366, and strong ions (>50% relative intensity) were observed at *m/z* 321, 320 and 274 due to loss from M^+ of ethoxide radical, ethanol and two ethanols, respectively. $LiAlH_4$ reduction followed by formation of the OTMS derivative for GC/MS demonstrated that the dicarboxylate function was converted into two OTMS-substituted alcohols as indicated by the following mass spectrum: 426 (2, M^+); 411 (1, $[M - CH_3]^+$); 369 (1, $[M - CH_3(CH_2)_3]^+$); 336 (49, $[M - TMSOH]^+$); 323 (7); 147 (23); 103 (99, $^+CH_2OTMS$); 73 (100, TMS).

A conjugated diene moiety was indicated in the dimethyl 2-butyl-3,5-tetradecadienedioate structure by its UV spectrum which showed a smooth-featureless spectrum typical of conjugated dienes at λ_{max} 235 *nm* ($\varepsilon = 25{,}800$). The diene was also demonstrated by hydrogenation of dimethyl 2-butyl-3,5-tetradecadienedioate which also confirmed the position of the butyl group as follows: 311 (5, $[M - CH_3O]^+$); 286 (9, $[M - (CH_2)_3CH_3 + H]^+$); 283 (24, $[M - COOCH_3]^+$); 269 (9); 254 (23); 222 (8); 154 (18); 130 (100, $[M - (CH_2)_{11}COOCH_3 + H]^+$); 98 (63); 87 (83); 55 (68). The strong McLafferty rearrangement ions at *m/z* 286 and 130 placed the butyl group at C-2. Further confirmation of the structure, particularly of the McLafferty rearrangement ions, was obtained by transesterification of the hydrogenation product by Na ethoxide which gave the following ions: 370 (0.3, M^+);

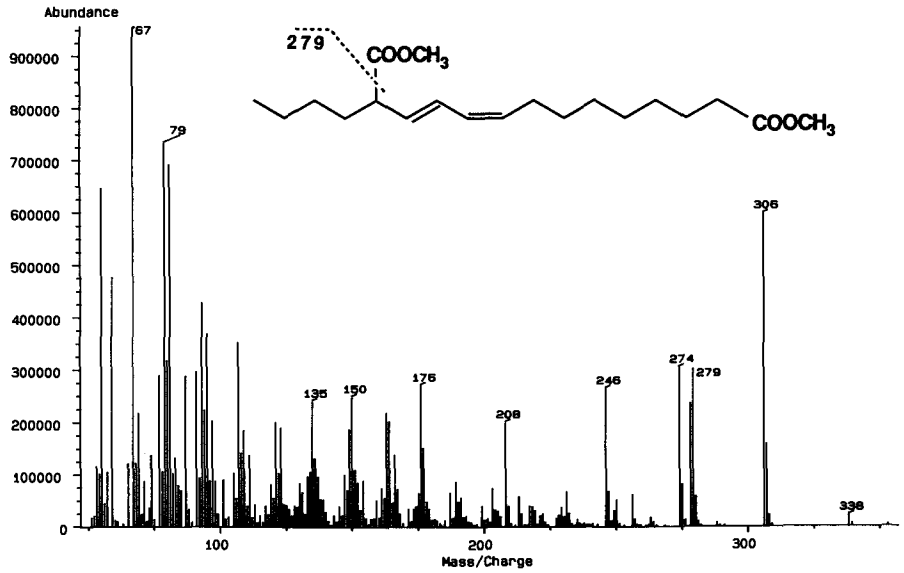

FIG. 4. Mass spectrum of dimethyl 2-butyl-3,5-tetradecadienedioate.

325 (11, $[M - CH_3CH_2O]^+$); 314 (12, $[M - (CH_2)_3CH_3 + H]^+$); 298 (12); 297 (56, $[M - COOCH_2CH_3]^+$); 283 (14); 268 (18); 227 (13, $^+(CH_2)_{11}COOCH_2CH_3$); 144 (100, $[M - (CH_2)_{11}COOCH_2CH_3 + H]^+$); 101 (70); 98 (59); 55 (65).

Dimethyl 2-butyl-3,5-tetradecadienedioate was subjected to ozonolysis, followed by $LiAlH_4$-reduction and formation of the OTMS ether. This treatment afforded two major peaks by GC/MS. The early eluting peak gave mass spectrum consistent with the *bis*-trimethylsilyloxy ether of 2-butyl-1,3-propanediol as follows: 261 (2, $[M - CH_3]^+$); 186 (17, $[M - TMSOH]^+$); 171 (39, $[M - TMSOH - CH_3]^+$); 147 (100, $TMSO^+=Si(CH_3)_2$); 143 (36); 133 (21); 129 (69); 103 (13, $^+CH_2OTMS$); 73 (90, TMS). The later eluting peak was identified as the *bis*-trimethylsilyloxy ether of 1,9-nonanediol from the following mass spectrum: 289 (1, $[M - CH_3]^+$); 214 (2, $[M - TMSOH]^+$); 199 (7, $[M - TMSOH - CH_3]^+$); 177 (10); 149 (36); 147 (89, $TMSO^+=Si(CH_3)_2$); 103 (33, $^+CH_2OTMS$); 83 (77); 75 (89); 73 (81, TMS); 69 (100); 55 (69). These fragmentation data placed the diene moiety of 2-butyl-3,5-tetradecadienedioic acid between C-3 and C-6. This result was confirmed by formation of a different derivative of the ozonolysis products; essentially, dimethyl 2-butyl-3,5-tetradecadienedioate was reduced by $LiAlH_4$, derivatized by MC chloride, ozonized and then methyl esterified for GC/MS (data not shown).

2-Butyl-4-hydroxy-5-tetradecenedioic acid, a third novel product of 13*S*-HPOD, was obtained by reaction of 13*S*-HPOD with 5 N KOH for 4 h. The compound was isolated as its dimethyl ester in 2.0% yield by LPLC followed by TLC purification (Table 2). Dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate was characterized by GC/MS of its OTMS ether and other chemically transformed derivatives. As shown in Figure 5, the GC/MS of the dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate OTMS ether afforded characteristic fragmentation ions of straightforward interpretation, but also the McLafferty rearrangement ion at *m/z* 130 $[129 + H]^+$ was observed. Rather strong ions at *m/z* 396 $[M - CH_3OH]^+$ and 369 $[M - COOCH_3]^+$ were also obtained.

$LiAlH_4$ reduction of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate followed by GC/MS of the OTMS ether furnished the following characteristic ions indicating the possibility of three OTMS ether moieties: 516 (0.1 M^+); 501 (0.2, $[M - CH_3]^+$); 426 (1, $[M - TMSOH]^+$); 413 (3, $[M - CH_2OTMS]^+$); 403 (4); 345 (4); 329 (100, $^+CHOTMSCH=CH(CH_2)_7CH_2OTMS$); 172 (19); 157 (13); 147 (14); 129 (24); 103 (10, $^+CH_2OTMS$); 73 (55, TMS). Further structural information was obtained by hydrogenation of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate followed by GC/MS of the OTMS ether. As can be seen by the following ions, the most prominent were from fragmentation vicinal to the C-11 OTMS ether: 415 (10, $[M - CH_3]^+$); 399 (9, $[M - CH_3O]^+$); 301 (25, $^+CH_2CHOTMS(CH_2)_9COOCH_3$); 287 (60, $^+CHOTMS(CH_2)_9COOCH_3$); 277 (12); 245 (100, $[M - (CH_2)_9COOCH_3]^+$); 185 (44, $^+(CH_2)_9COOCH_3$); 129 (12, $CH_3(CH_2)_3CH^+(COOCH_3)$); 127 (15); 73 (39, TMS).

Dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate was reduced by $LiAlH_4$, converted into an MC derivative, subjected to oxidative ozonolysis, and then methyl esters were formed. One fragmentation product, the MC derivative of methyl 9-hydroxynonanoate (retention time of 11.9 min), was identified by its *m/z* of 339 (0.8, $[M - CH_3O]^+$) and 233 (18), the latter ion, $[M - 137]^+$, being a characteristic of MC derivatives. The other fragment, a *bis*-MC derivative $CH_3(CH_2)_3CH(CH_2OMC)CH_2CHOMCCOOCH_3$, separated as diastereomers at 27.8 and 28.4 min, and both peaks, as expected, gave a small ion at *m/z* 431 (0.4 and 1, $[M - 137]^+$) as well as an intense one at *m/z* 293 (40 and 41, $[M - 137 - 137]^+$). These data placed the double bond of 2-butyl-4-hydroxy-5-tetradecenedioic acid at Δ9.

It was envisioned that 2-butyl-4-hydroxy-5-tetradecenedioic acid or its dimethyl ester could be converted by 5 N KOH into 2-butyl-3,5-tetradecadienedioic acid *via* elimination of the 11-hydroxyl group. However, after a 4 h incubation of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate at 35°C in 5 N KOH and separation of the methyl esterified products by TLC using Solvent A, it was

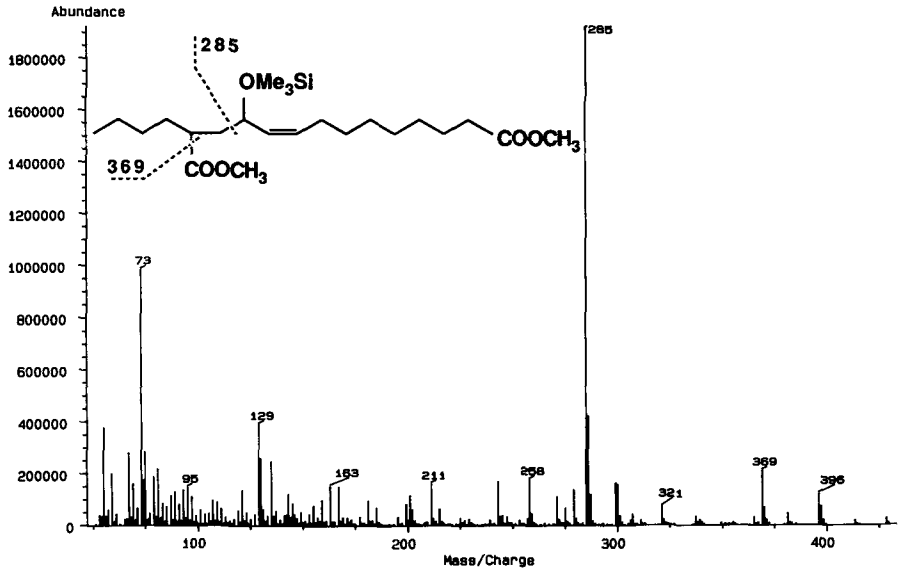

FIG. 5. Mass spectrum of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate, 11-trimethylsilyloxy ether.

determined that about two-thirds of dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate ($R_f = 0.25$) was converted into a less polar product consistent with formation of a γ-lactone ($R_f = 0.48$). No evidence for dimethyl 2-butyl-3,5-tetradecadienedioate was found. That methyl 2-butyl-4-hydroxy-5-tetradecenedioate γ-lactone was the principal compound isolated from the incubation mixture was shown by GC/MS as follows: 324 (4, M^+); 306 (11, $[M - H_2O]^+$); 293 (18, $[M - CH_3O]^+$); 292 (48, $[M - CH_3OH]^+$); 278 (17); 265 (11); 264 (13); 208 (20); 167 (29, $[M - (CH_2)_7COOCH_3]^+$); 139 (30); 95 (39); 81 (48); 67 (65); 55 (100). The γ-lactone methyl ester was hydrogenated and examined by GC/MS giving the following diagnostic ions: 295 (39, $[M - CH_3O]^+$); 294 (25, $[M - CH_3OH]^+$); 280 (9); 270 (21, $[M - CH_3(CH_2)_3 + H]^+$, McLafferty rearrangement); 266 (10); 251 (13); 238 (14); 235 (15); 197 (19); 141 (100, $[M - (CH_2)_9COOCH_3]^+$); 98 (52); 95 (71); 69 (77); 55 (97). Methyl ester formation of the hydrogenated γ-lactone reaction mixture revealed that the main component was a hydrogenolysis product (from elimination of the 11-hydroxyl) which afforded a GC/MS virtually identical to that of hydrogenated dimethyl 2-butyl-3,5-tetradecadienedioate, that is, dimethyl 2-butyl-tetradecanedioate. Finally, isolated methyl 2-butyl-4-hydroxy-5-tetradecenedioate γ-lactone was reconverted to dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate by treatment with 5 N KOH for 3.5 h at 35°C followed by reesterification. Conversion to the OTMS ether followed by GC/MS afforded virtually the same spectrum as shown in Figure 5. Also, evidence was obtained that a portion of 2-butyl-4-hydroxy-5-tetradecenedioic acid (free dioic acid) was partially converted into γ-lactone after normal work-up in the absence of KOH.

Conversion of the oxoepoxyoctadecenoic acid. About 175 μg methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate was treated with 1 mL 5 N KOH at 35°C taking aliquots at 30, 60, 120 and 240 min. Very little, if any, conversion of this compound occurred in the samples taken at early times. However, at 240 min, methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate was almost completely converted into roughly equivalent amounts of 2-butyl-3,5-tetradecadienedioic acid and 2-butyl-4-hydroxy-5-tetradecenedioic acid as determined by TLC of their dimethyl esters after treatment with diazomethane. The esters isolated from the TLC plates were examined by GC/MS (dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate as the OTMS ether), and these spectra afforded data virtually identical to those compounds obtained directly from KOH treatment of 13*S*-HPOD.

The data shown in Figure 2 implies that in KOH-13*S*-HPOD reactions, the intermediate (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid was converted into 2-butyl-3,5-tetradecadienedioic acid and 2-butyl-4-hydroxy-5-tetradecenedioic acid with greater ease than by the direct incubation of its methyl ester. This may be due to the insolubility of the methyl ester in aqueous KOH. Methyl esters are often remarkably resistant to saponification, because of the absence of a less polar solvent, like ethanol, to effect solvation.

Minor products. Other minor products were examined by GC/MS after TLC isolation from LPLC fractions (Table 2). These minor products were considerably different in both structure and polarity.

Of the minor products, mixed isomeric dienols other than 13*S*-HOD comprised about 2% of the total after 4 h reaction (Table 2), and these compounds were seen by TLC as a more polar shoulder of the main 13*S*-HOD peak (Fig. 1). As determined by GC/MS ion intensity, about half to less than half of this fraction was comprised of methyl (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoate (OTMS ether) judging from its retention time by GC/MS (11.8 min) and its mass spectrum: 382 (38, M^+); 311 (25); 225 (100). Since the minor isomer obtained by oxidation of linoleic acid by soybean lipoxygenase is (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (1-5%), the corresponding hydroxy fatty acid probably originated from the hydroperoxide present as a minor component in the 13*S*-HPOD used as a reactant. The next most abundant component

(ranging from about 25% to almost half of the dienols) proved to be an unusual product giving a retention time (9.8 min) and mass spectral data (as its methyl ester, OTMS ether) consistent with hydroxyheptadecadienoic acid with the dienol located between C-9 and C-13 as follows: 368 (40, M^+), 311 (36, $[M - CH_3(CH_2)_3]^+$); 211 (75, $[M - (CH_2)_7COOCH_3]^+$); 130 (33); 73 (100, TMS). Hydrogenation gave, not only a significant amount of its hydrogenolysis product, methyl heptadecanoate (data not shown), but a product shown by GC/MS of its OTMS ether to be methyl 11-hydroxyheptadecanoate as follows: 357 (3, $[M - CH_3]^+$); 341 (6, $[M - CH_3O]^+$); 325 (19, $[M - CH_3OH - CH_3]^+$); 287 (100, $[M - CH_3(CH_2)_5]^+$); 258 (13); 187 (71, $[M - (CH_2)_9COOCH_3]^+$); 73 (50, TMS). Therefore, the GC/MS data of both the hydrogenated and the unhydrogenated compound together showed that the product was 11-hydroxy-9,12-heptadecadienoic acid, a nonconjugated dienol. These data were consistent with the mass spectra of OTMS ethers of a similar nonconjugated dienol, methyl 11-hydroxy-9,12-octadecadienoate and its product of hydrogenation (10). The remainder of the dienols, methyl (*E,E*)-9- or (*E,E*)-13-hydroxyoctadecadienoates, were present in very minor amounts. Their mass spectra were similar to the corresponding (*E,Z*)-dienols with retention times of 12.8 and 13.2 min for the 9- and 13-OTMS ethers, respectively.

A minor product, isolated by TLC with the fraction containing dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate, was identified as methyl 11-hydroxy-12,13-epoxy-9-octadecenoate by GC/MS of its OTMS ether. This compound, a commonly observed decomposition product of 13*S*-HPOD, eluted after 13.5 min, compared to 13.8 min for dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate (OTMS ether), and gave a mass spectrum consistent with published data (5).

A number of minor polar compounds were present (Table 2). The weak TLC band immediately below dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate ($R_f = 0.20$) appeared to be its isomer with a similar mass spectrum of its OTMS ether and with a slightly longer GC/MS retention time (14.4 min *vs.* 13.8 min for dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate, OTMS ether). More polar than the aforementioned compound was a weak TLC band ($R_f = 0.15$) that appeared to be dimethyl 3-hydroxy-4-tridecenedioate by GC/MS of its OTMS ether (GC/MS retention time of 8.8 min) as follows: 358 (1, M^+); 343 (13, $[M - CH_3]^+$); 326 (4, $[M - CH_3OH]^+$); 311 (18, $[M - CH_3OH - CH_3]^+$; 298 (7); 285 (55, $[M - CH_2COOCH_3]^+$); 237 (19); 236 (15); 201 (31, $[M - (CH_2)_7COOCH_3]^+$); 135 (30); 89 (81); 73 (100, TMS). $LiAlH_4$ reduction of dimethyl 3-hydroxy-4-tridecenedioate followed by formation of the *tris*-OTMS ether gave the mass spectrum: 446 (0.3, M^+); 403 (5); 356 (6, $[M - TMSOH]^+$); 329 (100, $[M - (CH_2)_2$-OTMS$]^+$); 217 (13, $TMSO^+{=}CHCH{=}CHOTMS$); 155 (75); 147 (46, $TMSO^+{=}Si(CH_3)_2$); 129 (33); 103 (36, $^+CH_2OTMS$); 73 (95, TMS). The latter derivative was further hydrogenated and examined by GC/MS as the *tris*-OTMS ether affording diagnostic fragment ions as follows: 433 (5, $[M - CH_3]^+$); 331 (31, $[M - (CH_2)_2OTMS]^+$); 219 (89, $[M - (CH_2)_{10}OTMS]^+$); 177 (7); 147 (65, $TMSO^+{=}Si(CH_3)_2$); 103 (99, $^+CH_2OTMS$); 73 (100, TMS). The most polar of the TLC fractions obtained near the origin contained a small quantity of methyl trihydroxyoctadecenoate as identified by GC/MS of its *tris*-OTMS ether (data not shown) as well as other unidentified compounds.

Several minor products were isolated from TLC fractions less polar than 13*S*-HPOD (Table 2). One of these, methyl 13-oxo-9,11-octadecadienoate, was found as a minor component of fractions containing methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate and dimethyl 2-butyl-3,5-tetradecadienedioate, apparently migrating with intermediate or similar polarity to the former two compounds. The amount of ketodiene appeared to be variable in these fractions; the GC/MS peak(s) attributed to the ketodiene isomers were usually nearly absent but could occasionally amount to 25% of the total ion intensity. The ketodiene was identified by its characteristic *m/z* ions at 308, M^+, 277 $[M - CH_3O]^+$, 237 $[M - CH_3(CH_2)_4]^+$, and 151 $[M - (CH_2)_7COOCH_3]^+$.

The least polar compound migrating near the front in TLC (Fig. 1) could not be identified as it would not elute by GC/MS. Another minor component eluting between the unknown and dimethyl 2-butyl-3,5-tetradecadienedioate gave a mass spectrum consistent with methyl 13-methoxy-9,11-octadecadienoate, which undoubtedly originated as an artifact by etherification during the diazomethane treatment and/or the extraction using aqueous methanol.

A TLC fraction migrating immediately below the methyl (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoate fraction contained several short-chain compounds which had GC/MS retention times between 6 and 8 min. These compounds gave mass spectra consistent with dimethyl dienedioates of 10-, 12- and 13-carbon chain lengths (Table 2). These compounds were not examined further to confirm their putative structures.

DISCUSSION

It has been known for many years that organic hydroperoxides (1), including lipid hydroperoxides (2), are converted into the corresponding alcohols by alkali treatment. Recently, the alkali conversion of 13*S*-HPOD and 13*S*-HPOT has been studied in more detail revealing that the conversion resulted in retention of the stereochemistry of the reactant hydroperoxide (4). In this study we confirm that the stereoconfiguration is conserved by a completely independent method based on GLC separation of MC derivatives. We also show by GC/MS and IR spectra that the main product is identical to $NaBH_4$-reduced 13*S*-HPOD, that is, 13*S*-HOD. By the use of ^{14}C-tracer, it was found that the overall conversion of 13*S*-HPOD to 13*S*-HOD is close to 75%.

The origin of the remaining 25% of products was investigated. (9*Z*)-13-Oxo-*trans*-11,12-epoxy-9-octadecenoic acid was the most abundant of these by-products, but it was formed to a maximum of about 7% after a 40-min reaction. It was demonstrated that (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid could be further converted into 2-butyl-3,5-tetradecadienedioic acid and 2-butyl-4-hydroxy-5-tetradecenedioic acid accounting for the transient appearance of the former. This latter transformation is fully consistent with the known Favorskii rearrangement (11,12), which proceeds through a cyclopropanone intermediate. In the base-catalyzed rearrangement of 2,3-epoxycyclohexanones, such as piperitone oxide and isophorone oxide, transformations occurred that

were analogous to those giving rise to 2-butyl-3,5-tetradecadienedioic acid, 2-butyl-4-hydroxy-5-tetradecenedioic acid, and the γ-lactone of the latter (12). In the previous study it was postulated that the γ-lactone eliminated to form the product analogous to 2-butyl-3,5-tetradecadienedioic acid (12); however, in the present work this conversion was not observed. The data are consistent with the formation of 2-butyl-3,5-tetradecadienedioic acid directly from the cyclopropanone intermediate. Thus, the formation of products can be largely explained as shown in Figure 6.

The γ-lactone of 2-butyl-4-hydroxy-5-tetradecenedioic acid (methyl ester) was only observed after dimethyl 2-butyl-4-hydroxy-5-tetradecenedioate was incubated with KOH; however, a small amount of the γ-lactone was detected after work-up of the free dioic acid. A minor amount of the γ-lactone could have escaped observation after incubation of 13*S*-HPOD with KOH, but it would not be expected to be formed in KOH solution. It is possible the formation of γ-lactone could have been favored by transesterification of the dimethyl ester in KOH.

Several of the other minor products could also originate from Favorskii rearrangement of (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid. For example, 11-hydroxy-9,12-heptadecadienoic acid could arise from the cyclopropanone intermediate by elimination of the carbonyl group as CO_2 after oxidation. Previously, the decarbonylation of cyclopropanones into olefins has been observed to occur in the presence of basic H_2O_2 (13). Also, the lack of a 2-butyl group and one additional carbon is the only structural feature that distinguishes 3-hydroxy-4-tridecenedioic acid from 2-butyl-4-hydroxy-5-tetradecenedioic acid implying the elimination of the butyl group plus a carbonyl from the intermediate. Several other short-chain dioic acids could arise from the Favorskii rearrangement, but their mechanism of formation is less obvious.

The stability of 13*S*-HOD to exposure to 5 N KOH for rather long periods is remarkable, but even more surprising was the survival of the nonconjugated, double allylic hydroxy fatty acid, 11-hydroxy-9,12-heptadecadienoic acid, in alkali. Similar fatty acids are notably unstable to acid treatment, but are stable in the absence of acids (10).

The mechanism by which KOH replaces hydroperoxide with hydroxyl without affecting the stereochemistry should be considered. It seems unlikely that the mechanism would involve homolytic cleavage of the hydroperoxide oxygens as the alkoxyl radical derived from 13*S*-HPOD is known to largely rearrange into the 12,13-epoxyallylic radical which then combines with O_2 at C-9 and C-11 (14). 12,13-Epoxy-11-hydroxy-octadecenoic acid was found, but it was quantitatively insignificant. As suggested previously (3), one possibility is the nucleophilic displacement from the distal OH of the hydroperoxide by hydroxide anion affording 13*S*-HOD anion and H_2O_2; however, in this very alkaline medium one would surmise that the hydroperoxide group would exist principally as an anion which would repulse approach of the hydroxide anion. Perhaps this could explain why the KOH reaction is relatively slow when compared to a more rapid conversion of 13*S*-HPOD to the corresponding dienol by thiolate anion under mildly alkaline conditions (15). Previous work (4) showed total loss of peroxide oxygen when the conversion was complete after 3 h, but H_2O_2 may have a transient existence during the first 60–100 min of hydroperoxide decomposition by KOH. This H_2O_2 may be the active oxygen species responsible for formation of the epoxide group of (9*Z*)-13-oxo-*trans*-11,12-epoxy-9-octadecenoic acid.

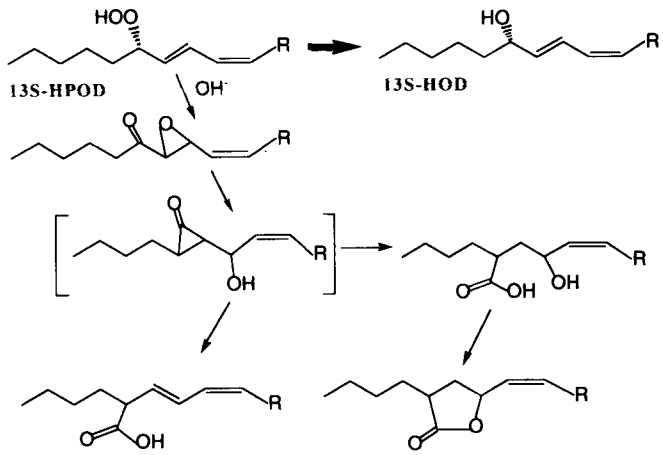

FIG. 6. Proposed pathway to 13*S*-HOD, as well as other products *via* Favorskii rearrangement; R = $-(CH_2)_7COOH$. The cyclopropanone fatty acid within brackets is proposed as an intermediate, but it was not directly observed. Abbreviations as in legend of Figure 1.

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Dynamics of Iron-Ascorbate-Induced Lipid Peroxidation in Charged and Uncharged Phospholipid Vesicles

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Peroxidation of egg yolk phosphatidylcholine (egg PC) liposomes was induced by addition of ascorbic acid (AsA) and Fe(II) in the presence of a trace of autoxidized egg PC (PC-OOH), but not in the absence of PC-OOH. PC-OOH was degraded upon addition of AsA and Fe(II) but not of either one alone. The results suggest that PC-OOH is necessary to initiate lipid peroxidation by AsA/Fe(II). AsA oxidation in the bulk water phase was also associated with an increase in lipid peroxidation by AsA/Fe(II) in the presence of PC-OOH, but not in the absence of PC-OOH. Furthermore, the spin probe 12-NS [12-(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid], which labels the hydrophobic region of dimyristoyl phosphatidylcholine (DMPC) liposomal membranes, was degraded upon addition of AsA and Fe(II) in the presence of PC-OOH, but not in the absence of PC-OOH. These results indicate that the "induction message" that is associated with decreases of PC-OOH and AsA in the initiation step of lipid peroxidation must be transferred from the membrane surface to the inner hydrophobic membrane region. AsA in the bulk phase was oxidized faster and more extensively upon its addition together with Fe(II) to egg PC liposomes than to DMPC liposomes, though the initial content of PC-OOH in the former was 5–10 times lower than in the latter. This suggests that, in egg PC liposomes, the OOH-groups of new PC-OOH generated in the inner membrane regions must become accessible from the surface, enabling reaction with AsA/Fe(II) which in turn would result in an extensive decrease in AsA. By contrast, in DMPC liposomes, that do not generate PC-OOH, AsA is only oxidized slightly in connection with the degradation of the PC-OOH initially present. The effect of surface charges on the membrane surface was also studied to obtain further information on the initiation step of lipid peroxidation. The rate of lipid peroxidation by AsA/Fe(II) or Fe(III) decreased in the order, egg PC liposomes ≫ negatively charged egg PC liposomes containing dicetylphosphate > positively charged egg PC liposomes containing stearylamine. The rate of associated AsA oxidation was in the order, egg PC liposomes ≫ egg PC/stearylamine liposomes > egg PC/dicetylphosphate liposomes. However, in DMPC liposomes that do not generate PC-OOH, the rates of AsA oxidation associated with the reductive cleavage of PC-OOH by AsA/Fe(II) and coupled with the reduction of Fe(III) to Fe(II) were in the order, DMPC liposomes = DMPC/stearylamine liposomes ≫ DMPC/dicetylphosphate liposomes. These differences in the rates of lipid peroxidation, depending on differences in membrane charge, are discussed in relation to two properties of AsA: (i) its antioxidant property through trapping of lipid radicals and (ii) its prooxidant properties (a) by being an effective iron chelator thus altering the reactivity of iron with oxygen and peroxides and (b) by being an iron reductant and providing a source of Fe(II).

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Lipid peroxidation has received much attention both as it relates to pathological events in biological systems and to food deterioration (1–5). Peroxidation usually proceeds by the following free-radical chain reactions:

$$LH \rightarrow L\cdot \quad \text{(initiation reactions)}$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad \text{(propagation reaction)}$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad \text{(propagation reaction)}$$

where LOO· is the lipid peroxy radical and LOOH is the lipid hydroperoxide. Iron is supposed to be the catalyst of the initiation reaction. Iron and ascorbic acid (AsA) are well known to initiate lipid peroxidation, and indeed they are often used for this purpose in model experiments (6–9). However, it is unknown how these water-soluble substances can initiate the reactions occurring in the inner hydrophobic membrane core. Furthermore, AsA action appears to be a complex process as AsA can act on lipid oxidation both as prooxidant and antioxidant. Its antioxidant effect is supposed to scavenge lipid free radicals formed during lipid peroxidation. It is also not clear how water-soluble AsA can reduce radicals generated in the inner hydrophobic membrane core.

Recently, Thomas *et al.* (10) reported that the removal of LOOH from membranes by treatment with phospholipid hydroperoxide glutathione peroxidase effectively inhibited lipid peroxidation by xanthine-xanthine oxidase plus Fe(III) or AsA plus Fe(III). We (11) also observed that a trace of preformed LOOH played an important role in iron-induced lipid peroxidation and proposed a site-specific initiation mechanism in which the binding of iron to the membrane surface is a prerequisite for its reaction with the hydroperoxy group of LOOH. The alkoxyl radical (LO·) formed by a Fenton-like reaction [LOOH + Fe(II) → LO· + Fe(III) + OH^-] at the surface may penetrate into the hydrophobic region and trigger the initiation reaction:

$$LOOH + Fe(II) \rightarrow LO\cdot + Fe(III) + OH^-$$

$$LO\cdot + LH \rightarrow LOH + L\cdot \quad \text{(initiation reaction)}$$

In this paper, we report studies on the requirement of LOOH for initiation of lipid peroxidation induced by AsA and Fe(II) and discuss the dynamics of peroxidation of phospholipids in bilayer membranes.

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Abbreviations: AsA, ascorbic acid; DMPC, dimyristoyl phosphatidylcholine; ESR, electron spin resonance; egg PC, egg yolk phosphatidylcholine; HEPES, 2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; 12-NS, 12(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid; MDA, malondialdehyde; PC-OOH, hydroperoxide of egg PC; TBA, thiobarbituric acid.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (egg PC) was obtained from Nippon Oil and Fats Co. (Tokyo, Japan). The preparation was contaminated with 3.8 μmol of hydroperoxide of egg PC (PC-OOH)/mmol egg PC, as determined iodometrically by the method of Buege and Aust (12). Dimyristoyl phosphatidylcholine (DMPC), *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES), dicetylphosphate, superoxide dismutase (from bovine erythrocytes) and catalase (from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). 12-(*N*-Oxyl-4,4'-dimethyloxazolidin-2-yl)stearic acid (12-NS) was from Aldrich Chemical Co. (Milwaukee, WI). α,α'-Dipyridyl and triphenylphosphine were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Ascorbyl-6-palmitate, L-AsA, $FeSO_4$ and $FeNH_4(SO_4)_2$ were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

Preparation of hydroperoxides. PC-OOHs were prepared as described previously (13). Briefly, a sample of 100 mg of PC dissolved in 5 mL of methanol containing 0.1 mM methylene blue was shaken continuously under illumination from a 30-W tungsten projection lamp at room temperature for 12 h. The PC-OOH obtained was purified by high-performance liquid chromatography (HPLC) on a reverse-phase glass column (240 × 10 mm) prepacked with Lichloprep RP-8 (Merck, Darmstadt, Germany; silica gel powder binding octane, 40–63 mm size) using chloroform/methanol/water (1:10:0.5, by vol) as eluant. The PC-OOH obtained was a mixture of conjugated and non-conjugated isomers. The purity of the PC-OOH, as determined iodometrically (12), was 98.2 mol%.

Preparation of liposomes. Liposomes were prepared as described previously (7) with a minor modification. Stock solutions of egg PC and DMPC in chloroform with or without stearylamine or dicetylphosphate were evaporated under nitrogen. The thin lipid film was dispersed in 10 mM HEPES buffer, pH 7.0, and then subjected to ultrasonic irradiation in a Bransonic-12 sonic bath (Yamato, Tokyo, Japan) at 40°C. When necessary, egg PC was freed of contaminating PC-OOH by treatment with triphenylphosphine in chloroform (11) just before preparation of liposomes. Residual triphenylphosphine was not removed because PC-OOH was produced again during the processes of its removal and during liposome preparation. For preparation of 12-NS-labeled DMPC liposomes, a solution of 3.6 μmol of DMPC, 0.1 μmol of 12-NS and 0 or 0.4 μmol of PC-OOH in chloroform was evaporated under nitrogen, and the resulting film was dispersed in 1.0 mL of 10 mM HEPES buffer (pH 7.0) and sonicated (14).

Lipid peroxidation assay. Lipid peroxidation was measured by monitoring oxygen consumption with a Clark-type oxygen electrode as described previously (15). Oxygen consumption was calculated assuming an oxygen concentration of 217 nmol/mL in the initial incubation mixture at 37°C. Lipid peroxides formed during incubation were also measured by the thiobarbituric acid (TBA) method as described previously (7) and expressed as TBA-reactive substances as equivalents in nmol of malondialdehyde/μmol egg PC. The incubation conditions and the concentrations of constituents in reaction mixtures are given in the legends of the figures and tables.

Measurement of oxidation of ascorbic acid. Oxidation of AsA was measured at 37°C by monitoring the decrease in absorbance at 265 nm as described by Buettner (16). The molar absorption of AsA at pH 6.1 is reported to be 14,500 $M^{-1} \cdot cm^{-1}$ at 265 nm (16). The colorimetric method of Murata *et al.* (17) with slight modification was used to determine the concentration of AsA.

Electron spin resonance (ESR) measurement. 12-NS, an *N*-oxyl-4,4'-dimethyloxazolidine derivative of stearic acid, was used as a spin probe to label the hydrophobic region of liposomal membranes as described above. The reaction was started by addition of 5 μL of 20 mM AsA and then 5 μL of 2 mM $FeSO_4$ to 1 mL of 12-NS-labeled liposomes. After incubation at 25°C, the reaction mixture was transferred to a flat rectangular ESR cell. The ESR spectra of 12-NS were recorded under the following conditions (11): microwave power, 8 mW; magnetic field, 3374 G; scanning range, 25 G; modulation width, 1.0 G.

RESULTS

As shown in Figure 1, addition of AsA plus Fe(II) [AsA/Fe(II)] to liposomes prepared from egg PC contaminated with a trace of PC-OOH (0.38 mol%, measured before preparation of liposomes) induced lipid peroxidation (line 1). Lipid peroxidation induced by AsA/Fe(II) was slightly suppressed in liposomes prepared from egg PC pretreated with 5 μM triphenylphosphine (18) to reduce PC-OOH to the corresponding alcohol (PC-OH) (line 2), and was completely suppressed in liposomes prepared from egg PC pretreated with 8 μM triphenylphosphine (line 3). This suppression was reversed by addition of 3 μM PC-OOH to egg PC pretreated with 8 μM triphenylphosphine (line 4). Addition of ferrous ions alone did not promote lipid peroxidation (line 5), though a high concentration of ferrous ions induced slight peroxidation (line 6). TBA-reactive substances were also measured to confirm the formation of lipid peroxides (see legend to Fig. 1). The amounts of TBA-reactive substances formed and oxygen consumed (lines 1–5) were well correlated. Fenton reagent [Fe(II) and H_2O_2] did not induce peroxidation of egg PC. The addition of α-tocopherol, ascorbyl palmitate, a lipid-soluble analog of AsA, or a high concentration (>1 mM) of AsA completely inhibited oxygen consumption associated with lipid peroxidation by AsA/Fe(II), but superoxide dismutase, catalase or OH-radical scavengers such as thiourea and mannitol were not inhibitory (data not shown), as reported by Bachowski *et al.* (19).

We measured AsA oxidation associated with lipid peroxidation induced by AsA/Fe(II) in the presence and absence of PC-OOH. As shown in Figure 2 (line 1), AsA was rapidly oxidized upon its addition together with Fe(II) to egg PC liposomes not treated with triphenylphosphine, as measured by a decrease in absorbance at 265 nm (16). The ultraviolet-absorbance of AsA could not be measured in triphenylphosphine-treated egg PC liposomes, because triphenylphosphine has a strong absorbance at 265 nm. So oxidation of AsA in egg PC liposomes treated with triphenylphosphine to remove PC-OOH was measured colorimetrically with α,α-dipyridyl (17). Table 1 shows that AsA was not oxidized in egg PC liposomes from which PC-OOH had been removed by treatment with triphenylphosphine, but was oxidized extensively in egg PC liposomes not treated with triphenylphosphine. We compared

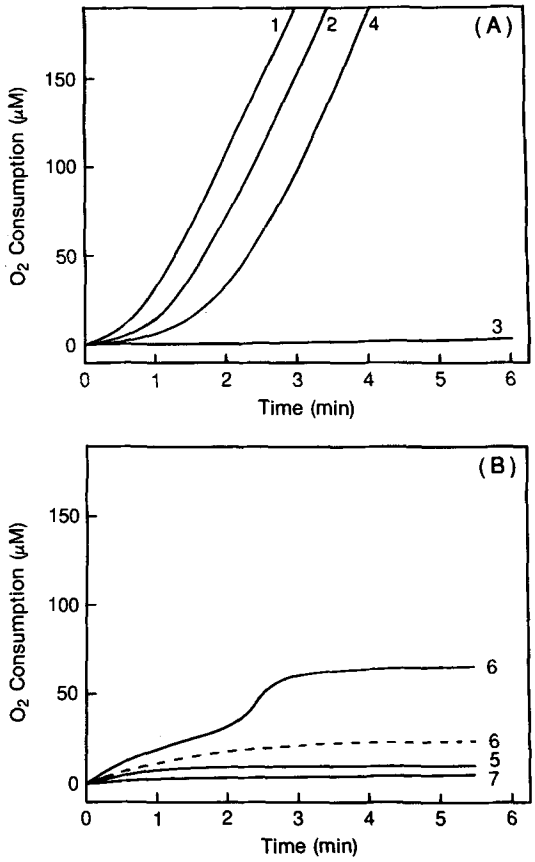

FIG. 1. Hydroperoxide of egg PC (PC-OOH)-dependent peroxidation in egg yolk phosphatidylcholine (egg PC) liposomes induced by ascorbic acid (AsA) and Fe(II), and its inhibition by antioxidants. Reaction 1—egg PC (not treated with triphenylphosphine, contaminated with 5–8 μM PC-OOH/mM egg PC), AsA and Fe(II); Reaction 2—egg PC (treated with 5 μM triphenylphosphine), AsA and Fe(II); Reaction 3—egg PC (treated with 8 μM triphenylphosphine), AsA and Fe(II); Reaction 4—egg PC (treated with 8 μM triphenylphosphine), PC-OOH (3 μM), AsA and Fe(II); Reaction 5—egg PC (not treated with triphenylphosphine) and Fe(II); Reaction 6—egg PC (not treated with triphenylphosphine) (continuous line) or dimyristoyl phosphatidylcholine (DMPC) (dotted line) and Fe(II) (110 μM) and Reaction 7—egg PC (not treated with triphenylphosphine), AsA, Fe(II) and α-tocopherol (6 μM) or ascorbyl palmitate (20 μM). The increases of thiobarbituric acid-reactive substances after incubation for 10 min were: Reaction 1—133.9, Reaction 2—128.4, Reaction 3—0.1, Reaction 4—120.0 and Reaction 5—6.6 nmol malondialdehyde/mL reaction mixture. The concentrations of reactants were 1 mM egg PC or DMPC, 100 μM AsA, 10 μM $FeSO_4$ and 10 mM 2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (pH 7.0). Reactions were started by addition of $FeSO_4$ to the reaction mixtures at 37°C.

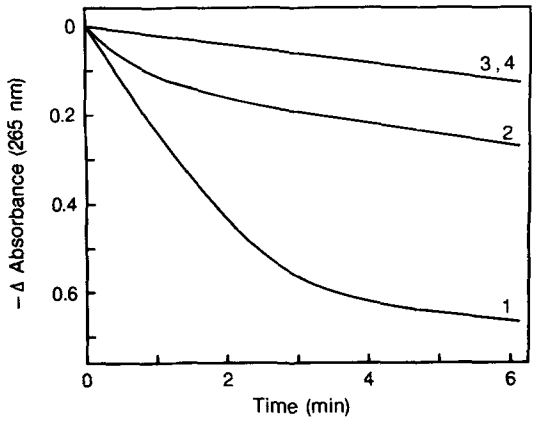

FIG. 2. Oxidation of ascorbic acid (AsA) in the presence of Fe(II) in egg yolk phosphatidylcholine (egg PC) and dimyristoyl phosphatidylcholine (DMPC) liposomes with and without hydroperoxide of egg PC (PC-OOH). Reaction 1—egg PC liposomes not treated with triphenylphosphine, Reaction 2—DMPC liposomes containing PC-OOH, Reaction 3—DMPC liposomes, Reaction 4—liposome-free system. Reaction mixtures contained 1 mM egg PC (contaminated with 5–8 μM PC-OOH) or DMPC with or without 50 μM PC-OOH, 100 μM AsA, 10 μM $FeSO_4$ and 10 mM 2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (pH 7.0). Incubation conditions were as for Figure 1.

the oxidation rates of AsA in egg PC liposomes that generate lipid peroxides and in DMPC liposomes that do not. AsA was oxidized more rapidly upon addition of AsA/Fe(II) to egg PC liposomes contaminated with less than 10 μM PC-OOH than upon addition of AsA/Fe(II) to DMPC liposomes containing 50 μM PC-OOH. Moreover, oxidation of AsA was higher in DMPC liposomes with PC-OOH than in those without PC-OOH (Fig. 2 and Table 1).

Table 2 shows the effects of Fe(II), AsA, and of both on the degradation of PC-OOH in DMPC liposomes.

TABLE 1

Oxidation of AsA by Fe(II) in Liposomes with Different Charges with and without PC-OOH

Liposome system[a]	Decrease of AsA (μM/5 min)
DMPC (without PC-OOH)	6.6
DMPC (with 10 μM PC-OOH)	14.4
DMPC (with 50 μM PC-OOH)	25.0
Egg PC	50.1
Egg PC (with 0.2 mM dicetylphosphate)	7.5
Egg PC (with 0.2 mM stearylamine)	10.6
Egg PC (pretreated with 10 μM triphenylphosphine)	4.5

[a]System contained 1 mM dimyristoyl phosphatidylcholine (DMPC) or 1 mM egg yolk phosphatidylcholine (egg PC) with or without indicated additions or pretreatments, and 100 μM ascorbic acid (AsA), 10 μM $FeSO_4$ and 10 mM 2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (pH 7.0). Reactions were carried out for 5 min at 37°C. Egg PC not treated with triphenylphosphine was contaminated with 0.38 mol% of hydroperoxide of egg PC (PC-OOH).

TABLE 2

Degradation of PC-OOH in DMPC Liposomes by AsA/Fe(II)

Incubation system[a]	Degradation of PC-OOH[b] (%)
AsA	4.9
Fe(II)	5.3
AsA/Fe(II)	60.5

[a]The concentration of hydroperoxide of egg yolk phosphatidylcholine (PC-OOH) was 100 μM. The concentrations of other reactants were as for Figure 2. Incubations were carried out at 37°C for 5 min. AsA, ascorbic acid.

[b]Degradation of PC-OOH was determined by high-performance liquid chromatography. DMPC, dimyristoyl phosphatidylcholine.

PC-OOH was degraded only in the presence of both AsA and Fe(II).

Next, we investigated how the prooxidant effect of water-soluble AsA/Fe(II) is transmitted to the hydrophobic initiation site of lipid peroxidation within the membrane. Labeled nitroxide radicals in liposomal (14) or micellar (11) lipid aggregates have been reported to be lost by reacting with radicals formed during membrane lipid peroxidation. The spectrum of 5-NS exhibits wide, highly anisotropic bands, whereas that of 12-NS shows a set of three symmetrical peaks (Fig. 3) indicating a higher fluidity of its environment deep within the membrane in which 12-NS is located. Table 3 shows the consumption of 12-NS, possibly due to reaction with radicals generated deep within the membrane. The ESR signals of 12-NS were decreased by AsA/Fe(II) in DMPC liposomes with PC-OOH, but not appreciably in those without PC-OOH. The addition of AsA or Fe(II) individually did not cause any decrease in the intensity of the ESR spectrum of 12-NS in DMPC liposomes containing PC-OOH (Table 3).

As shown above, PC-OOH was necessary to initiate lipid peroxidation by AsA/Fe(II). The hydroperoxy (OOH) group has been reported (20,21) to have a large dipole moment and thus to be likely to be close to the membrane surface where it can react with water-soluble AsA/Fe(II). Hence, we next investigated the effects of different surface charges of liposomes on lipid peroxidation by AsA/Fe(II) and the associated oxidation of AsA, degradation of PC-OOH and reduction of Fe(III) to Fe(II) by AsA.

Figure 4(A) shows that lipid peroxidation by AsA/Fe(II) was induced markedly in uncharged egg PC liposomes, weakly in negatively charged liposomes containing dicetylphosphate, and not appreciably in positively charged liposomes containing stearylamine. Addition of AsA plus Fe(III) also initiated lipid peroxidation, but AsA/Fe(III) had a lower initiation ability than AsA/Fe(II). The data in Figure 4B and Table 1 illustrate the oxidation of AsA associated with lipid peroxidation by AsA/Fe(II) in differently charged liposomes. AsA was oxidized rapidly in uncharged egg PC liposomes, but not oxidized appreciably

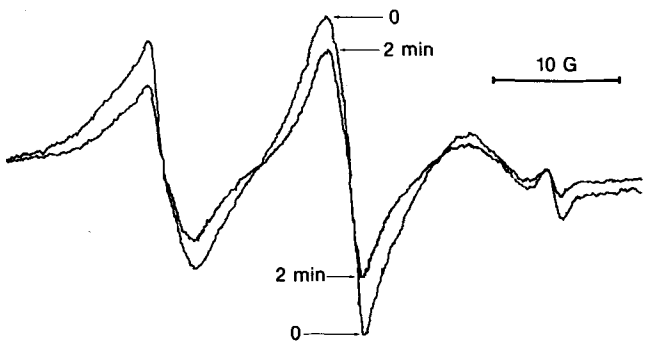

FIG. 3. Decrease of electron spin resonance signal of 12-(*N*-oxyl-4,4′dimethyloxazolidin-2-yl)stearic acid (12-NS) by ascorbic acid (AsA)/Fe(II) in dimyristoyl phosphatidylcholine (DMPC) liposomes with hydroperoxide of egg yolk phosphatidylcholine (PC-OOH). The figure shows the 12-NS spectra before and 2 min after addition of AsA and Fe(II) to DMPC liposomes containing PC-OOH (see also Table 3).

TABLE 3

Decrease of Electron Spin Resonance Signal of 12-NS by AsA/Fe(II) in DMPC Liposomes with or without PC-OOH (see Fig. 3)[a]

	Decrease of 12-NS signal (%)
PC-OOH + AsA/Fe(II)	28.4
PC-OOH + Fe(II)	3.8
PC-OOH + AsA	3.2
AsA/Fe(II)	6.6
Fe(II)	2.5
AsA	1.8

[a]The concentrations of reactants were 0.1 mM 12-(*N*-oxyl-4,4′-dimethyloxazolidin-2-yl)stearic acid (12-NS), 0.4 mM hydroperoxide of egg yolk phosphatidylcholine (PC-OOH), 3.6 mM dimyristoyl phosphatidylcholine (DMPC), 100 μM ascorbic acid (AsA) and 10 μM $FeSO_4$ in 10 mM 2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (pH 7.0). Incubations were carried out for 2 min at 37°C.

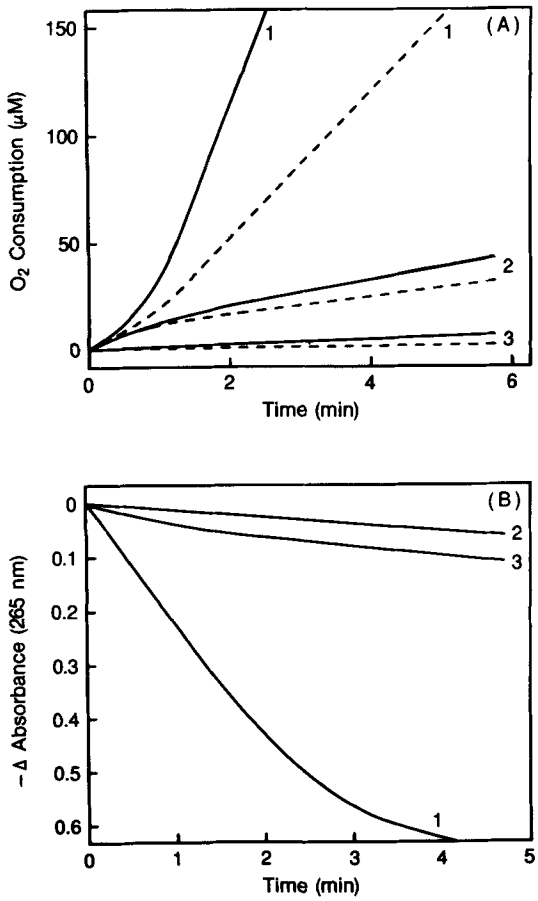

FIG. 4. (A) Lipid peroxidation by ascorbic acid (AsA) with Fe(II) or Fe(III) and (B) associated oxidation of AsA in uncharged egg yolk phosphatidylcholine (egg PC) liposomes, and liposomes negatively charged with dicetylphosphate, or positively charged with stearylamine. Reaction 1—egg PC liposomes, Reaction 2—egg PC/dicetylphosphate liposomes, Reaction 3—egg PC/stearylamine liposomes. The reaction mixtures contained 1 mM egg PC, 0.2 mM dicetylphosphate or stearylamine, 100 μM AsA, 10 μM $FeSO_4$ (solid lines) or $FeNH_4(SO_4)_2$ (dotted lines) and 10 mM 2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (pH 7.0). Other experimental conditions were as for Figure 1.

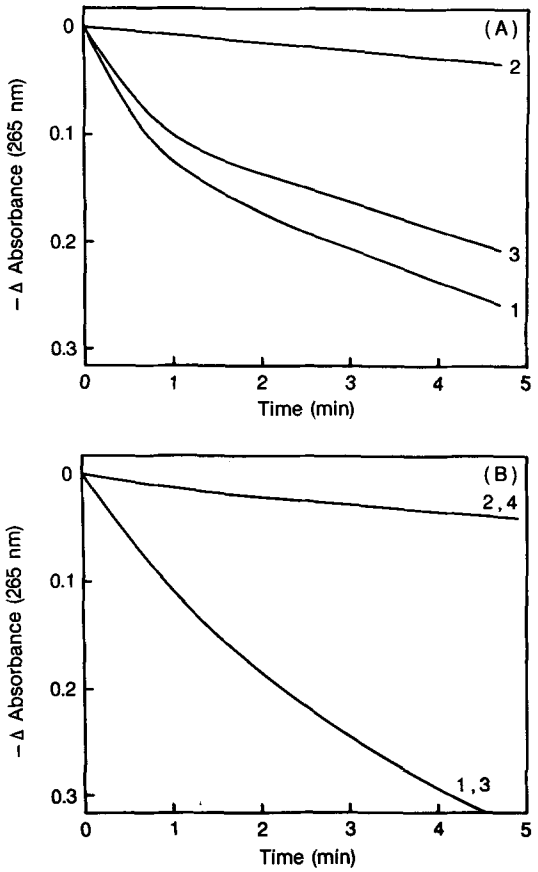

FIG. 5. Oxidation of ascorbic acid (AsA) (A) associated with reductive cleavage of hydroperoxide of egg PC (PC-OOH) by AsA/Fe(II) and (B) coupled with reduction of Fe(III) to Fe(II) in dimyristoyl phosphatidylcholine (DMPC) liposomes with and without dicetylphosphate or stearylamine. Reaction 1—DMPC liposomes, Reaction 2—DMPC/dicetylphosphate liposomes, Reaction 3—DMPC/stearylamine liposomes, Reaction 4—liposome-free system. The concentrations of PC-OOH were 50 and 0 μM in Experiments (A) and (B), respectively. The concentrations of other components were 1 mM DMPC, 0.2 mM dicetylphosphate or stearylamine, 100 μM AsA, 10 mM 2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (pH 7.0) and 10 μM $FeSO_4$ (Experiment A) or 25 μM $FeNH_4(SO_4)_2$ (Experiment B). Incubation conditions were as for Figure 1.

in egg PC liposomes charged by stearylamine or dicetylphosphate.

Next we investigated the effect of membrane charge on the ability of AsA/Fe(II) to initiate lipid peroxidation by measuring the rate of AsA oxidation associated with PC-OOH cleavage. For study of the initiation step of lipid peroxidation only, we used DMPC liposomes to avoid complications from PC-OOH generation during incubation. We found that the oxidation rate of AsA in the reaction of AsA with PC-OOH in the presence of Fe(II) in DMPC liposomes was almost the same as in DMPC/stearylamine liposomes, but faster than in DMPC/dicetylphosphate liposomes (Fig. 5A). We also examined the effect of membrane charges on the reduction of Fe(III) to Fe(II) by AsA. The relative rates of AsA oxidation in the liposomes were also in the order, DMPC liposomes = DMPC/stearylamine liposomes ≫ DMPC/dicetylphosphate liposomes (Fig. 5B).

DISCUSSION

We found that AsA/Fe(II) induced lipid peroxidation in liposomes prepared from fresh egg PC that was contaminated with a trace of PC-OOH. However, addition of AsA/Fe(II) did not induce lipid peroxidation in liposomes prepared from egg PC after removal of contaminated PC-OOH by pretreatment with triphenylphosphine. Moreover, AsA was oxidized only slightly by Fe(II) in the system with liposomes prepared from DMPC or egg PC treated with triphenylphosphine, but was appreciably oxidized in the system with DMPC liposomes containing PC-OOH and was markedly oxidized in the system with liposomes of egg PC not treated with triphenylphosphine (Table 1 and Fig. 2). These results suggest that PC-OOH is necessary to initiate lipid peroxidation by AsA/Fe(II).

In systems without PC-OOH, AsA was autoxidized slightly but significantly (1.4 μM/min) (Fig. 2, lines 3 and 4) associated with a slight oxygen consumption (0.5 μM/min). Iron-catalyzed autoxidation of AsA in a system of this type is reported to give rise to $O_2^{\cdot-}$ and ·OH (22,23). However, addition of AsA plus Fe(II) did not initiate lipid peroxidation in the absence of PC-OOH (Fig. 1, line 3), indicating that ·OH and $O_2^{\cdot-}$ generated during autoxidation of AsA/Fe(II) are not involved in the process of lipid peroxidation. This was supported by the findings that superoxide dismutase, catalase and ·OH scavengers did not inhibit lipid peroxidation by AsA/Fe(II).

Addition of AsA/Fe(II) to DMPC liposomes containing PC-OOH resulted in a decrease in the intensity of the spectrum of 12-NS, a spin probe monitoring the inner hydrophobic membrane region. However, the intensity of the 12-NS spectrum decreased only slightly upon omission of AsA, Fe(II) or PC-OOH from this system (Table 3 and Fig. 3). These results indicate that the alkoxy radical (PC-O·) generated from PC-OOH near the membrane surface by a Fenton-like reaction triggers the hydrophobic initiation reaction of lipid peroxidation. Lipid peroxidation was induced by AsA/Fe(II) much faster in uncharged egg PC liposomes than in negatively charged egg PC/dicetylphosphate liposomes (Fig. 4), indicating that the cleavage of PC-OOH by a Fenton-like reaction is catalyzed by the weakly charged Fe(II)-AsA complex, not by positively charged free Fe(II).

From these results, we propose a possible site-specific mechanism of lipid peroxidation in liposomes induced by the addition of AsA and Fe(II) (Fig. 6, where PC is a phosphatidylcholine with unsaturated fatty acid, and PC· is phosphatidylcholine with a fatty acid radical). The OOH-group of PC-OOH may be cleaved near the membrane surface by the Fe(II)-AsA complex (Reaction 1), and the resulting PC-O· may penetrate into the hydrophobic region of the membranes (2 in Fig. 6) and react with the unsaturated moieties of fatty acids of egg PC (Reaction 3) resulting in a chain reaction (Reaction 4). We found that the oxidation rate was much greater upon addition of AsA/Fe(II) to egg PC liposomes containing less than 10 μM PC-OOH (not treated with triphenylphosphine) than upon addition to DMPC liposomes containing 50 μM PC-OOH (Fig. 2 and Table 1). These results indicate that a lipid hydroperoxide is generated by the radical chain reaction in the inner hydrophobic regions of egg PC membranes (Reactions 3 and 4), and the polar OOH-group of new PC-OOH then moves towards the surface (5 in Fig. 6)

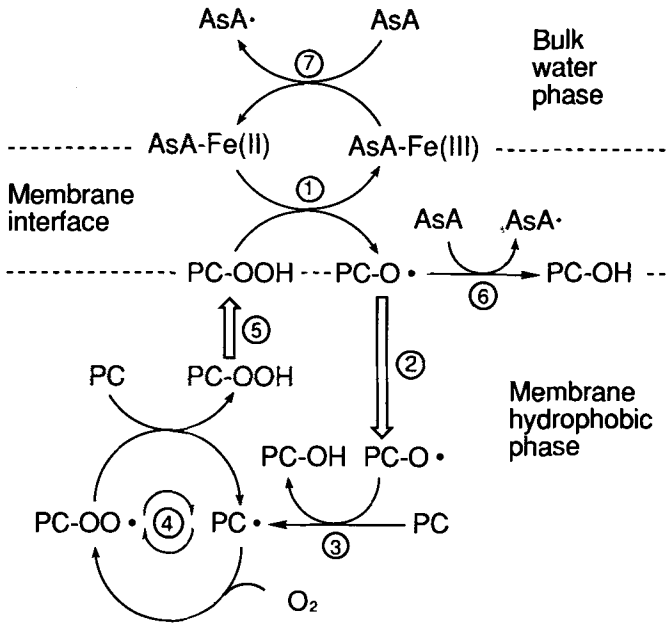

FIG. 6. Proposed mechanism of lipid peroxidation induced by ascorbic acid (AsA)/Fe(II) in liposomal membranes. PC-OOH, hydroperoxide of egg yolk phosphatidylcholine; AsA·, ascorbyl radical.

and reacts with the Fe(II)-AsA complex (Reaction 1). The resulting Fe(III) complex is then reduced by AsA to regenerate the Fe(II) complex (Reaction 7) resulting in further degradation of AsA.

Lipid peroxidation by AsA/Fe(II) was much slower in negatively charged liposomes containing dicetylphosphate than in uncharged liposomes (Fig. 4A, solid line 2). Moreover, we recently observed slow lipid peroxidation by AsA/Fe(II) in negatively charged egg PC liposomes containing phosphatidylserine (24). Slow oxidation of AsA associated with reductive cleavage of PC-OOH by AsA/Fe(II) (Figs. 4B and 5A, line 2) and with reduction of Fe(III) to Fe(II) (Fig. 5B, line 2) were also observed in dicetylphosphate-containing liposomes. A probable explanation for these findings is that positively charged Fe(II) or Fe(III) may interact with negatively charged dicetylphosphate and so react less with negatively charged AsA, thus reducing the formation of an AsA/Fe complex and initiation of lipid peroxidation.

By contrast, in positively charged liposomes containing stearylamine, the oxidation rates of AsA associated with reductive cleavage of PC-OOH by AsA/Fe(II) and coupled with reduction of Fe(III) to Fe(II) were high and almost the same as those in uncharged DMPC liposomes (Figs. 5A and B, line 3), but the rates of O_2 consumption associated with lipid peroxidation by AsA/Fe(II) and associated oxidation of AsA were much lower than those in uncharged egg PC liposomes (Figs. 4A and B, line 3). These results indicate that, in positively charged stearylamine liposomes, Reactions 1 and 2 both progress smoothly, but that the propagation step (Reaction 4) in lipid peroxidation does not progress, resulting in decreased formation of PC-OOH and consequently reduced oxidation of AsA (Reaction 1). Since negatively charged AsA was reported (25) to be present at the positively charged surface of egg PC/stearylamine liposomes and in the bulk water phase in uncharged egg PC or negatively charged egg PC/dicetylphosphate liposomes, it should effectively scavenge PC-O· (Reaction 6) at the membrane surface before the latter penetrates into the membrane resulting in a decrease in Reactions 3 and 4. Reaction 4 may also be reduced by scavenging of PC-OO· by AsA, because we recently observed that PC-OO· "floated up" from the hydrophobic region and was scavenged effectively by AsA at the surface of positively charged stearylamine liposomes but not of negatively charged dicetylphosphate liposomes. This idea is supported by the finding that ascorbyl palmitate, a lipid-soluble analog of AsA, strongly inhibited AsA/Fe(II)-induced lipid peroxidation in egg PC liposomes (Fig. 1), as its antioxidant polar group is located in the uncharged membrane surface (26).

Lipid peroxide and iron may play important roles in the initiation of lipid peroxidation *in vivo*. Recently, new types of antioxidants, such as a LOOH breakdown seleno compound (27) and an iron-inactivating chelator (28), were reported to prevent the pathological states induced by LOOH-linked and iron-dependent lipid peroxidation and free radical generation. The development of antioxidants other than radical scavenging types and studies on their effects seem important.

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Role of Lipid Structure in the Activation of Phospholipase A_2 by Peroxidized Phospholipids

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The time course of hydrolysis of a mixed phospholipid substrate containing bovine liver 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE) catalyzed by *Crotalus adamanteus* phospholipase A_2 was measured before and after peroxidation of the lipid substrate. The rate of hydrolysis was increased after peroxidation by an iron/adenosine diphosphate (ADP) system; the presence of iron/ADP in the assay had a minimal inhibitory effect. The rate of lipid hydrolysis was also increased after the substrate was peroxidized by heat and O_2. Similarly, peroxidation increased the rate of hydrolysis of soy PC liposomes that did not contain PE. In order to minimize interfacial factors that may result in an increase in rate, the lipids were solubilized in Triton X-100. In mixtures of Triton with soy PC in the absence of PE, peroxidation dramatically increased the rate of lipid hydrolysis. In addition, the rate of hydrolysis of the unoxidizable lipid 1-palmitoyl-2-[1-^{14}C]oleoyl PC incorporated into PC/PE liposomes was unaffected by peroxidation of the host lipid. These data are consistent with the notions that the increase in rate of hydrolysis of peroxidized PC substrates catalyzed by phospholipase A_2 is due largely to a preference for peroxidized phospholipid molecules as substrates and that peroxidation of host lipid does not significantly increase the rate of hydrolysis of nonoxidized lipids.
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The activity of phospholipase A_2 is increased in a number of tissues subjected to free-radical damage as a consequence of oxidative stress. Phospholipase inhibitors, such as chlorpromazine (1,2), inhibit the loss of phospholipid which is associated with peroxidative damage in cells. Extensive hydrolysis of cell membrane or lipoprotein phospholipids in the absence of adequate means for repair may lead to serious adverse consequences. For example, peroxidation of low density lipoproteins (LDL) and the associated hydrolysis of LDL phospholipids may play a role in the development of atherosclerosis (3). In addition, the structural reorganization of cell membranes consequent to lipid peroxidation and phospholipid hydrolysis may lead to edema (4), cell death and myocardial injury. Products of phospholipid hydrolysis, such as lysophospholipids (5), may also disrupt normal regulatory mechanisms that depend upon maintenance of the integrity of cell membrane structure. Adverse consequences of fatty acid and lysophospholipid accumulation have been demonstrated in brain and cardiac tissue in which the detergent effects of these molecules alter membrane function (5). In addition, release of arachidonic acid may produce eicosanoids that alter cardiovascular function, and phospholipid degradation has been observed in ischemia-reperfusion injury (6). However, control of lysolipid concentrations in membranes may be regulated by deacylation and reacylation of lipids. Accumulation of low concentrations of lysolipids has been shown to lead to increases in membrane permeability (7), activation of membrane-bound enzymes (8) and the activation of macrophages (9).

Preferential hydrolysis of peroxidized lipids has been observed in isolated brain capillaries (10), hepatic lysosomes (11), mitochondria (12) and microsomes (13) and in purified lipid mixtures (14,15). The rate of hydrolysis of these peroxidized lipids by phospholipase A_2 is some 2–3 times the rate of that of the unoxidized lipids in lipid mixtures that have been systematically examined (14). A repair mechanism based on this preference for peroxidized lipids has been proposed by van Kuijk *et al.* (16). The hydrolysis of peroxidized lipids results in excision of the peroxidized fatty acyl chains which are reduced, repaired and reesterified. This proposal is supported by several reports that demonstrate that phospholipase A_2 activity is required to release peroxidized fatty acids for subsequent detoxification by glutathione peroxidase (7,8, 17,18).

It is also conceivable that peroxidized lipids may promote the hydrolysis of neighboring unoxidized lipids, locally altering the membrane structure and possibly leading to cellular degeneration. Such a mechanism would suppose that peroxidation results in physical alterations in the substrate structure that lead to an increased rate of hydrolysis of the unoxidized lipids. A possible structural relationship between peroxidized lipids and increased activity of phospholipase A_2 was suggested by experiments of Sevanian *et al.* (19) who correlated an increase in microviscosity of diphenylhexatriene in liposomes following peroxidation with an increase in enzyme activity. In addition, an increase in the susceptibility of the lipids to fusion was observed following peroxidation suggesting a decrease in the stability of the liposomes.

In order to explore mechanisms that might explain the increase in rate of hydrolysis of peroxidized phospholipids by phospholipase A_2, the effect of lipid peroxidation on the rate of lipolysis in detergent-dispersed lipid and of a nonoxidizable radiolabeled substrate incorporated into liposomes comprising a host peroxidizable lipid were measured. The addition of detergent is expected to minimize differences in the surface structure of the substrates that may be due to lipid peroxidation. The host lipid experiments were designed to test whether the rate of hydrolysis of an unoxidized lipid increases when the host lipid is peroxidized.

MATERIALS AND METHODS

Lipid vesicles. To prepare lipid vesicles, bovine liver 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC; Type II-B, Sigma

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Abbreviations: ADP, adenosine diphosphate; LDL, low-density lipoproteins; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; TBARS, thiobarbituric acid reactive substances.

Chemical Company, St. Louis, MO) or soy phosphatidylcholine (Type III-S; Sigma) and bovine liver 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE; Type VII; Sigma) were mixed in chloroform at a 4:1 molar ratio. In some experiments, 5 mole% 1-palmitoyl-2-oleoyl PC (Avanti Polar Lipids, Birmingham, AL) or a trace quantity (0.1 μCi/mL of liposomes) of 1-palmitoyl-2-[1-^{14}C]oleoyl PC (58.0 mCi/mmol; New England Nuclear, Boston, MA) was included. The organic solvent was first dried with N_2, then under vacuum. The lipids were dispersed in 0.1 M KCl, 10 mM Tris-HCl, pH 7.6 at a concentration of 2.5 mM by incubating at 37°C with occasional vortexing for 1 h. The resulting liposomes were sonicated to clarity [15 min at setting 4 with a Branson (Branson Ultrasonic, Danbury, CT) 350 Sonifier operating on a 50% duty cycle] on ice under an argon atmosphere. These clear vesicles were stored at room temperature overnight to anneal (20). To prepare Triton/phospholipid mixtures (21), liposomes were prepared as described above without sonication at a concentration of 2.5 mM in 0.1 M KCl, 10 mM Tris-HCl, pH 7.6 containing 10 mM Triton X-100 (Ultragrade, LKB, Stockholm, Sweden). In some experiments additional Triton X-100 was added. The lipids were then suspended in the detergent-containing buffer by vortexing for 10 min.

The vesicles were peroxidized by one of three methods: (i) *Peroxidation by iron/adenosine diphosphate (ADP).* Vesicles (2.5 mM) were mixed with a fresh solution of 1.2 mM ADP and 0.2 mM $FeSO_4$ in water to attain a final concentration of either 20 μM $FeSO_4$ and 120 μM ADP or 10 μM $FeSO_4$ and 60 μM ADP. The vesicles were then incubated at 37°C for 30 min under air. (ii) *Peroxidation by iron (II)/iron (III).* A solution of 10 mM $FeCl_3$ and 11 mM ethylenediaminetetraacetic acid prepared in N_2-purged water was added to the vesicles to attain a concentration of 0.1 mM $FeCl_3$. Then a fresh solution of $FeSO_4$ (1 mg/mL water) was added to attain a concentration of 0.1 mM. (iii) *Peroxidation by heat and oxygen.* Prior to swelling the lipids in buffer, the tubes were flushed with oxygen, capped and heated at 60°C for up to 4 h. These peroxidized samples were stored at −20°C for up to 24 h. Then, buffer was added to the lipids and vesicles were prepared as described above. Prior to phospholipase measurements, aliquots were taken for measurement of thiobarbituric acid reactive substances (TBARS).

The rate of hydrolysis of lipids catalyzed by phospholipase was measured directly following peroxidation by the pH-stat technique or by measurements of released [^{14}C]oleic acid as described in detail by McLean *et al.* (21). The vesicles were diluted after peroxidation to 0.5 mM in 5 mL of 0.5 mM bicine, 0.1 M KCl, 10 mM $CaCl_2$, pH 8.0. The assay temperature was 37°C. Hydrolysis was initiated by addition of 3 μL of a 0.3 mg/mL solution of *Crotalus admanteus* phospholipase A_2 (Sigma) in standard buffer without calcium.

TBARS were measured by addition of 0.5 mL of sample to 0.1 mL of butylated hydroxytoluene (2%), followed by 1.5 mL each of 20% trichloroacetic acid and 0.67% thiobarbituric acid/0.05 N NaOH. Reaction proceeded for 30 min at 100°C. The tubes were then cooled, centrifuged for 15 min at 3000 rpm and transferred to plastic semimicro cuvettes. The difference in absorbance at 532 nm and 580 nm (to correct for light scattering) was measured in a Beckman DU-7 spectrophotometer (Fullerton, CA). The TBARS were calculated in units of malondialdehyde equivalents using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

RESULTS

Mixtures of PC and PE were chosen for evaluation of the effects of peroxidation on the rate of hydrolysis of phospholipids because of their abundance in biological membranes and the availability of previous data on similar mixtures (16). Unoxidized mixtures of bovine liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are hydrolyzed at a relatively slow rate by *Crotalus adamanteus* phospholipase A_2 at 37°C (Fig. 1). The rate is increased dramatically after peroxidation with iron and ADP. Because iron and ADP were not removed prior to assay, it is possible that the iron/ADP mixture directly affects the enzyme. Addition of iron and ADP to the assay mixture at the concentration at which it is present in the peroxidized samples inhibits the rate of hydrolysis slightly, so that the increased rate of hydrolysis observed in Figure 1 is not the result of a direct interaction of the iron and ADP with the enzyme. In a second set of experiments, dry lipids were peroxidized by heat and oxygen, to eliminate the presence of peroxidizing agents during the assay. This resulted in a slightly lower TBARS than with iron peroxidation, but peroxidation of the lipids by heat and oxygen increases the rate of lipid hydrolysis (Fig. 2), and the rate of hydrolysis increases progressively with time of incubation under oxygen at elevated temperature. The rate of hydrolysis of mixed PC/PE liposomes also depends upon the extent of lipid peroxidation and peaks after a time that depends on the particular substrate and experimental conditions under which it is examined (data not shown). With 10 μM $FeSO_4$ and 60 μM ADP at 37°C, the maximal rate occurs when the TBARS

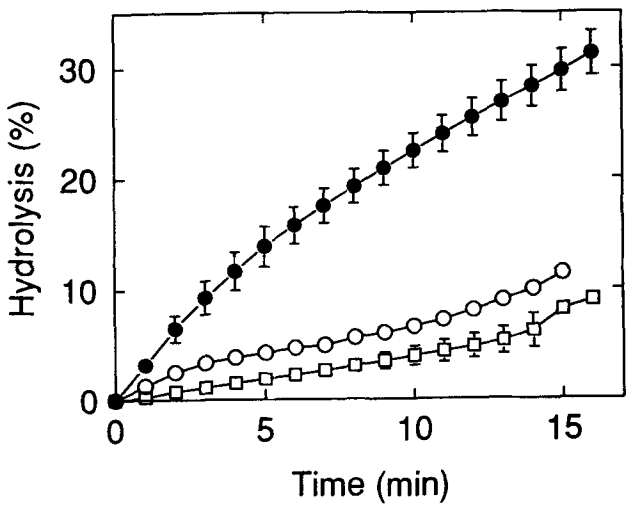

FIG. 1. Effect of peroxidation by iron adenosine diphosphate (ADP) on hydrolysis of 1,2-diacyl-*sn*-glycero-3-phosphocholine and phosphoethanolamine (PC/PE) (4:1) vesicles by phospholipase A_2. The rates of hydrolysis were measured by pH-stat (○) before incubation [thiobarbituric acid reactive substances (TBARS) of <0.2 nmol malondialdehyde (MDA)/μmole lipid]; (●) after incubation with 20 μM $FeSO_4$ and 120 μM ADP for 30 min at 37°C (TBARS of 7.9 ± 0.8 nmol MDA/μmole lipid); and (□) in the presence of 2 μM $FeSO_4$ and 12 μM ADP. Data are averages ± SEM of 3–4 experiments.

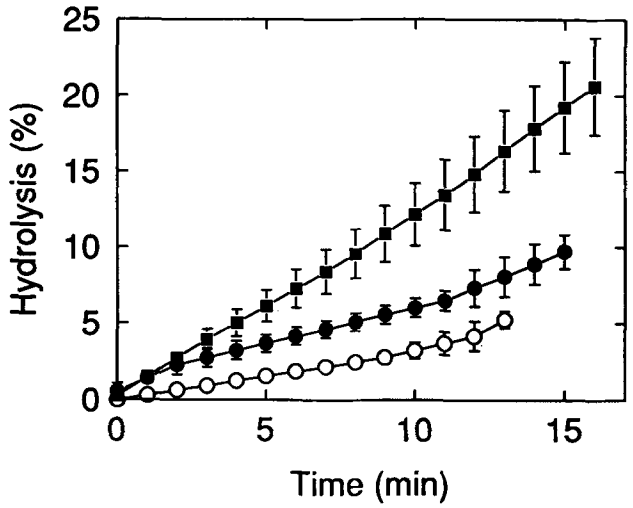

FIG. 2. Effect of incubation at elevated temperature under oxygen on the rate of hydrolysis of PC/PE vesicles by phospholipase A_2. Dry mixtures of PC/PE were incubated at 60°C for (○) 0 h (TBARS of <0.2 nmol MDA/μmole lipid), (●) 1 h (TBARS of 1.1 nmol MDA/μmole lipid) and (■) 4 h (TBARS of 2.1 nmol MDA/μmole lipid) prior to formation of vesicles and measurements of rates of hydrolysis. Abbreviations as in Figure 1.

reach ≈8 nmol/μmole lipid (Fig. 3). Further peroxidation of liposomal lipids as measured by TBARS formation results in slower rates of hydrolysis. Thus, the rate measured may depend critically on the extent of peroxidation. It is not possible to directly equate the TBARS results with the number of peroxidized lipid molecules.

One explanation for the increased rate of hydrolysis of peroxidized lipids in PC/PE mixtures is that the presence of the peroxidized lipids alters the structure of the vesicle in a manner that promotes interaction of the enzyme with substrate. One approach to test this hypothesis is to compare the rates of hydrolysis in the presence of a detergent which minimizes possible effects of substrate structure and physical form on the rates of hydrolysis. However, the presence of two types of phospholipids in the PC/PE mixture complicates interpretation of the data due to potential effects of the interface between the two lipids and the complications inherent in assessing experiments with more complex lipid mixtures. Thus, PC in the absence of PE was further investigated. Although more variability was observed in experiments with pure PC liposomes, the rate of hydrolysis of peroxidized PC liposomes exceeded that of the unoxidized liposomes (Fig. 4A) in a manner similar to that observed with the PC/PE mixtures. To minimize interfacial effects, samples were mixed with Triton X-100 at a molar ratio of 4:1 (Triton X-100 to lipid) and assayed. This addition of Triton to the peroxidized phospholipids resulted in a far more rapid rate of hydrolysis than with unoxidized PC mixtures. In the

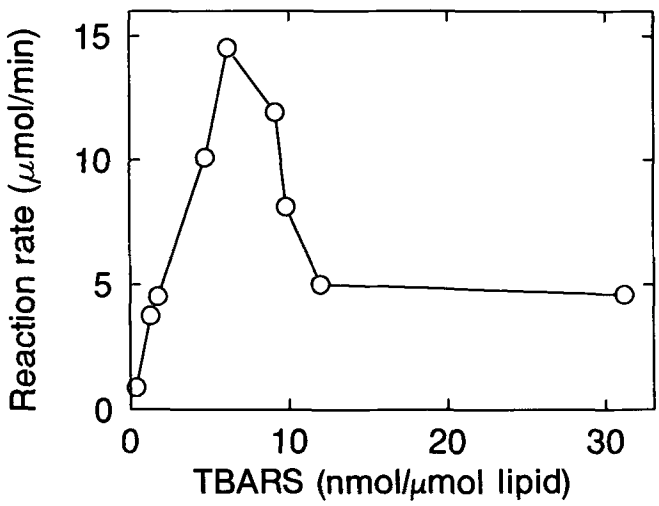

FIG. 3. Dependence of reaction rate on the extent of peroxidation as measured by TBARS. PC/PE (4:1) vesicles were peroxidized for various periods of time at 37°C with 10 μM $FeSO_4$ and 60 μM ADP. The rates were calculated from the initial linear portion of the kinetic course. Abbreviations as in Figure 1.

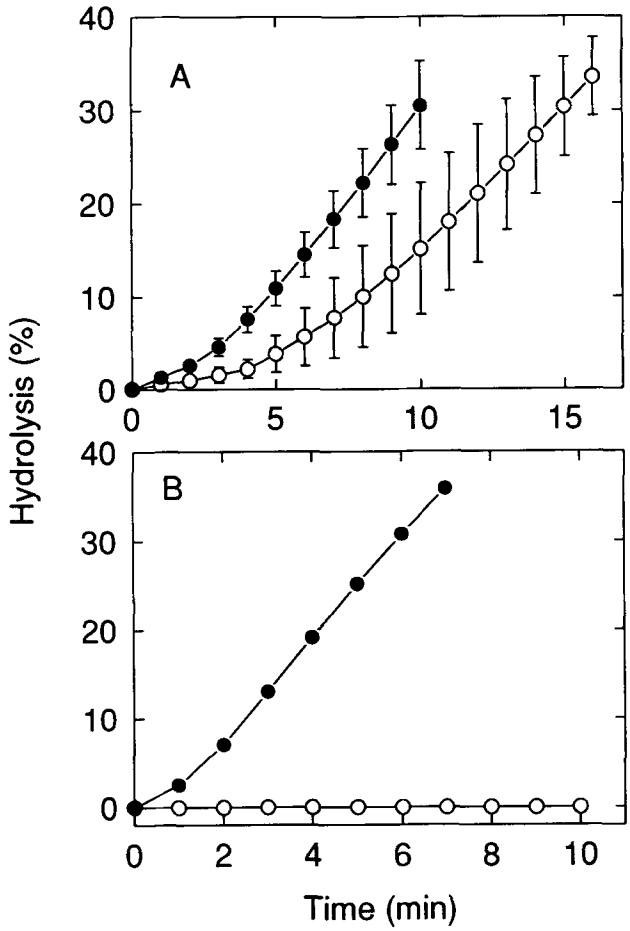

FIG. 4. Effect of peroxidation on hydrolysis of soy PC vesicles with and without Triton by phospholipase A_2. (A) Rates of hydrolysis of soy PC vesicles measured by pH-stat (○) before incubation (TBARS of 1.0 ± 0.1 nmol MDA/μmol lipid) and (●) after incubation with 100 μM Fe(II) and 100 μM Fe(III)/ethylenediaminetetraacetic acid for 30 min at 37°C (TBARS of 1.8 ± 0.2 nmol MDA/μmole lipid). Each curve is the average of two experiments. (B) Effect of peroxidation on rate of hydrolysis of soy PC in Triton X-100 micelles. Incubated (●) and unincubated (○) lipids from A were mixed with Triton X-100 (4:1 Triton/phospholipid mole/mole) and rates of hydrolysis were measured following addition of 3 μL (0.18 μg/mL) of enzyme. Abbreviations as in Figure 1.

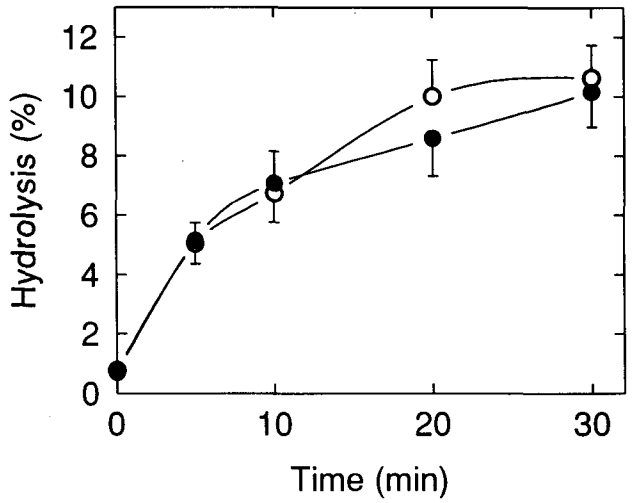

FIG. 5. Effect of peroxidation of host lipid on the rate of hydrolysis of 1-palmitoyl-2-[1-^{14}C]oleoyl PC by phospholipase A_2. PC/PE vesicles containing a trace amount of 1-palmitoyl-2-[1-^{14}C]oleoyl PC were peroxidized with iron/ADP as described in the legend to Figure 1. The rate of hydrolysis was measured by counting the released [^{14}C]oleic acid. (○) Unincubated vesicles (TBARS of 1.4 nmol MDA/μmol lipid), (●) incubated vesicles (TBARS of 8.7 nmol MDA/μmol lipid). Abbreviations as in Figure 1.

latter, no hydrolysis was measurable over a period of >60 min with a 4:1 ratio of Triton/PC (Fig. 4B). Higher ratios of Triton/PC were required to elicit measurable rates of hydrolysis in the unoxidized lipids, but peroxidized lipid/Triton mixtures were always more rapidly hydrolyzed (data not shown).

To test more directly whether peroxidation of the host lipid has any effect on the rate of hydrolysis of unoxidized lipids, the rate of hydrolysis of palmitoyl-[^{14}C]oleoyl PC in soy PC/soy PE vesicles before and after peroxidation was examined. Because palmitoyl-oleoyl PC is not readily peroxidized, it served as a marker for host lipid effects. The experiment described under Figure 1 was repeated with 1-palmitoyl-2-[1-^{14}C]oleoyl PC present in the liposomes, and the released fatty acids were assayed to determine the rate of hydrolysis of the radioactive palmitoyl-oleoyl PC tracer. In these experiments, peroxidation of the host lipid had no effect on the rate of hydrolysis of the palmitoyl-oleoyl PC (Fig. 5).

DISCUSSION

The present set of experiments demonstrates that peroxidation of mixed PC/PE vesicles by either iron/ADP or oxygen/heat results in a marked increase in the rate of phospholipase A_2-catalyzed hydrolysis that is not the result of a direct effect of peroxidizers on the enzyme. This supports the notion that phospholipase A_2 prefers peroxidized substrates (18,22). The dramatic increase in the overall rate of hydrolysis of PC/PE mixtures with peroxidation may be due to either a preference for phospholipid molecules with peroxidized acyl chains or a preference for structurally altered liposomes containing peroxidized lipids. It is difficult to distinguish between these two mechanisms in PC/PE mixtures because of potential differences in the rate of hydrolysis of the two components. The inability to make mechanistic conclusions with the mixed PC/PE mixture led us to study simple PC vesicles in more detail. To minimize interfacial interactions that may complicate interpretation of the effects of peroxidation of lipids on phospholipase activity, a detergent was included in some experiments. These experiments could not be interpreted at a molecular level with the PC/PE mixtures as addition of detergent may alter the substrate head-group preference of the enzyme (23,24).

In the simpler system containing PC in the absence of PE, the increase in rate of hydrolysis of lipids after peroxidation was relatively small. Addition of detergent increased the rate of hydrolysis of the lipids that had been incubated under peroxidizing conditions and reduced the rate of hydrolysis of unoxidized lipids. These data suggest that the peroxidized lipids are better substrates for the enzyme. An alternative explanation is that Triton enhances structural differences between the oxidized and peroxidized substrates. However, peroxidized lipids were still hydrolyzed more rapidly when the ratio of Triton to PC was increased. Consistent with this apparent substrate preference is the observation that the rate of hydrolysis of the nonoxidizable lipid, palmitoyl-[^{14}C]oleoyl PC, incorporated into a peroxidizable liposome was not influenced by peroxidation of host lipid.

These data suggest that in pure PC vesicles the primary effect of lipid peroxidation is to provide a phospholipid substrate with acyl chains that are preferentially hydrolyzed by the phospholipase. It does not appear that peroxidation of host lipid results in dramatic increases in the rate of hydrolysis of nonoxidized phospholipids. In terms of the proposed protective role of phospholipase A_2 in the repair of peroxidative membrane damage (16), the model provided by adding a trace of palmitoyl-oleoyl PC to peroxidizable lipids indicates that even in the case of transfer of nonoxidized lipids into regions of the membrane that have been damaged by peroxidation, the nonoxidized lipids may not be more rapidly hydrolyzed. However, the likelihood for peroxidation of lipid which diffuses into a region of peroxidized lipid is increased by its potential interaction with free radicals formed by peroxidative mechanisms at the site of injury. Such lipids would then be rapidly hydrolyzed in an attempt to minimize membrane damage.

No repair mechanism is available, to our knowledge, for the phospholipid hydrolysis associated with lipoproteins (25). We suggest that in cases of free-radical damage to lipoproteins or cells, in which the repair mechanism is unable to keep up with lipid hydrolysis, unchecked damage may occur that would lead to severe disruption of membrane or lipoprotein integrity and function. Part of this damage in LDL may be the result of transfer of liberated fatty acids from either the phospholipids or cholesteryl esters to amino groups on the surface of the lipoprotein (3). Recent experiments indicated that probucol prevents modification of the lipoprotein surface under conditions in which lipid peroxidation is observed, possibly by limiting transfer of fatty acids to the surface of the particle (26). These principles, when applied to the design of an antioxidant, suggest that one would need not only an effective free radical scavenger, but also a molecule that alters the structure of the membrane or lipoprotein surface in such a way that the transfer of nonoxidized lipids into the region of membrane peroxidative damage is limited. The possibility that other antioxidants may

exert their effects in a similar beneficial manner is currently under investigation.

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Structure-Function Relationships of Alkyl-Lysophospholipid Analogs in Selective Antitumor Activity

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This investigation was initiated in order to delineate the structure-function relationship of the anticancer alkyl-lysophospholipids and assess their degree of selective cytotoxicity toward neoplastic cells. A series of glycerol phosphocholine analogs with varying substitutions in the *sn*-1 and *sn*-2 position were tested for their inhibitory activity as measured by thymidine incorporation, clonogenic assays and effects on protein kinase C activity against a series of human leukemic cell lines and healthy bone marrow progenitor cells. The IC_{50} was determined for each of the compounds in each cell line and healthy bone marrow cells following a 4-h incubation. The data indicated that a 16–18 carbon chain at the *sn*-1 coupled with a short substitution at *sn*-2 had the broadest antitumor activity and was the least toxic to normal bone marrow cells. The results provide a number of useful leads toward the design and development of potentially more active phospholipid compounds.
Lipids 28, 511–516 (1993).

Since the discovery by Munder *et al.* (1) that synthetic analogs of naturally occurring lysophosphatidylcholine (LPC) had antitumor activity, considerable interest has focused on this new family of compounds which mediate their effects through cell surface events. Andreesen *et al.* (2), Modolell *et al.* (3) and others (4,5) stressed the selective cytotoxicity aspects. We have shown that there is a selective cytotoxic effect of synthetic alkyl-lysophospholipids (ALP) on neoplastic tissues and a sparing of normal marrow progenitor cells (NMPC) (6,7), and have begun clinical studies using one of these compounds as an *in vitro* purging agent to eliminate any residual leukemic cells from marrow obtained from patients in remission prior to autologous bone marrow transplantation (8).

A series of analogs have been synthesized, and from earlier studies it is evident that specific structural configurations are important for antitumor activity. Munder *et al.* (9) found that longer hydrocarbon chains (18 carbons) were more active than shorter (12 carbon) chains. Andreesen *et al.* (2) reported that an ether linkage of the aliphatic side chain in *sn*-1 of the glycerol molecule and substitution of the hydroxy group in the *sn*-2 position were essential for antitumor activity. Stereoisomers were also active, and greater growth inhibition was observed with choline phospholipid analogs than the ethanolamine phospholipid analogs (10). Replacement of oxygen with sulfur in *sn*-1 has yielded essentially an equally active product (11,12).

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Abbreviations: ALP, alkyl-lysophospholipid; BCS, bovine calf serum; BFU-E, erythroid bursts; CFU-E, erythroid colonies; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; GEMM, mixed colonies; GM-CFU, granulocyte-macrophage colonies; LP, lysophosphatidylcholine; NMPC, normal marrow progenitor cells; PHA-LCM, phytohemagglutinin-lymphocyte conditioned medium; PKC, protein kinase C.

The mechanism of action of ALP is not completely understood. The site of action appears to be the cell membrane. Evidence for this is the membrane damage seen with electron microscopy (13), inhibition of phosphatidylcholine synthesis (14), inhibition of protein kinase C (PKC) activity (15) and inhibition of Na, K-ATPase activity (16). In addition, DNA synthesis is indirectly inhibited (17). It is not known whether or not this is a direct effect or a result of cell surface modification induced by these compounds. Furthermore, it is not clear how these compounds are relatively selectively toxic to neoplastic tissues and spare healthy cells. It has been assumed that healthy cells are capable of metabolizing the compound, whereas neoplastic cells are not. It has been shown that healthy tissues contain an enzyme which cleaves the alkyl group in the *sn*-1 position, and tumor tissues lack this cleavage enzyme (18–20). However, one of the most active compounds, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) is not a substrate for the cleavage enzyme (21,22).

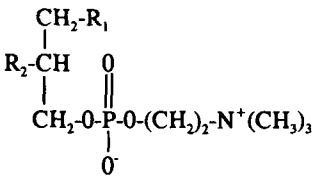
In order to gain further insight into structure-function relationships, we have tested a series of glycerophospholipid analogs for cytotoxicity against leukemic cell lines, effects on PKC activity and DNA synthesis. We chose a 4-h incubation time as preliminary studies with ET-18-OCH_3 indicated an anti-leukemic effect on the clonogenicity and thymidine incorporation of fresh leukemic cells, with little activity against clonogenic bone marrow cells. In addition, such a time interval would serve as practical methods for a clinical marrow purging procedure.

MATERIALS AND METHODS

Compounds. Figure 1 lists the compounds tested. Compounds A (ET-18-OCH_3) and L (BM 41.440, Boehringer Mannheim, Mannheim, Germany) were obtained from Dr. Wolfgang Berdel (Freie Universität Berlin, Berlin, Germany). The other compounds were synthesized by one of us (J.H.) as described previously (23,24). Synthesis of compounds C, D, G_1 and G_2 will be reported separately. All compounds were fully characterized, including satisfactory elemental analyses (within 0.3% of the calculated values). The compounds were stored at −20°C. Prior to testing for their cellular effects, stock solutions were prepared by dissolving the compounds in the appropriate medium, usually RPMI 1640, at a concentration of 1 mg/mL. The solutions could be frozen without loss of activity.

Human cell lines. The myeloid leukemic cell line HL60 was obtained from Dr. Robert Gallo (NIH, Bethesda, MD) (25); K562 was obtained from Bismark Lozzio (University of Tennessee, Knoxville, TN) (26); B-cell leukemic cell lines, Daudi and U-937 (human histiocytic lymphoma) with monocyte-like characteristics were obtained from American Type Culture Collection (Rockville, MD) (27). Cells were continuously cultured in RPMI 1640 medium and 10% bovine calf serum (BCS) (Hyclone, Logan, UT). Studies were conducted with cells during log-phase growth.

GLYCEROPHOSPHOCHOLINES



	R_1	R_2	
A	$O\text{-}(CH_2)_{17}CH_3$	OCH_3	(ET-18-OCH_3)
B	$O\text{-}(CH_2)_{17}CH_3$	$NHCOCH_3$	
C	$O\text{-}(CH_2)_{17}CH_3$	$NHCO\text{-}OCH_3$	
D	$O\text{-}(CH_2)_{17}CH_3$	$NHSO_2\text{-}CH_3$	
E	$O\text{-}(CH_2)_{15}CH_3$	$NHCO(CH_2)_{14}CH_3$	
G_1	$O\text{-}(CH_2)_{17}CH_3$	CH_3	
G_2	$O\text{-}(CH_2)_{17}CH_3$	CH_3	
H	$O\text{-}(CH_2)_{15}CH_3$	$NHCOCH_3$	
I	$O\text{-}(CH_2)_{15}CH_3$	OCH_3	
J	$O\text{-}(CH_2)_{15}CH_3$	SCH_3	
K	$S\text{-}(CH_2)_{15}CH_3$	$NHCOCH_3$	
L	$S\text{-}(CH_2)_{15}CH_3$	$CH_2O\text{-}CH_3$	(BM 41.440)
N	$O\text{-}(CH_2)_{15}CH_3$	$NHCOCF_3$	

FIG. 1. Structure of alkyl-lysophospholipid analogs. R_1 substitutions have either oxygen or sulfur linked to a 16 or 18 carbon chain. R_2 substitutions include methoxy (compounds A & I) acetamide (B, H & K), methoxycarbonylamino group (C), methansulfonamide group (D), a carbonyl group with a long carbon chain (E), 2-deoxy analogs (G_1 & G_2), which are chiral, G_1 is the *sn*-3 phosphocholine derivative and G_2 is the *sn*-1 phosphocholine, a thiomethyl group (J), methoxymethyl (L) and trifluoroacetyl group (N).

NMPC. Cells were obtained from donors for allogeneic bone marrow transplants (having given informed consent) by aspirate from posterior iliac crests or from bone marrow fragments following orthopedic procedures. Mononuclear cells were separated by Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co., St. Louis, MO) and the interphase cells were used in the assay.

Cytotoxicity assays. Varying concentrations of the compounds were incubated with 1×10^6 cells in 1 mL in RPMI 1640, 10% BCS (except for NMPC, where 10% fetal bovine serum, FBS, with 2×10^6 cells is used), 1% PS antibiotic mixture and 1% glutamine (Gibco, Grand Island, NY) (RPMI-C) at 37°C for 4 h. Following incubation, the cell line cells were diluted with RPMI-C to provide a suitable plating cell number and thereby diluting the drug at least 250-fold. For NMPC, the drug was effectively diluted by adding 4 mL of RPMI-C, pelleting, removing 4.8 mL media and resuspending to 1×10^6/mL with RPMI-C. Viability was determined by trypan blue dye exclusion at the end of the 4-h incubation.

Tritiated thymidine incorporation. For this assay, 4×10^5 cells in 200 μL of medium containing 10% BCS were incubated in triplicate with 0.4 μCi of methyl-[^{3}H]thymidine (spec. act. 80–90 Ci/mmol) for 1 h in 5% CO_2 at 37°C. Following incubation, the cells were harvested in a cell harvester (Brandel, Gaithersburg, MD), dried, scintillation fluid was added and the mixture was counted in a scintillation counter (17). The results were expressed as cpm/4×10^5 cells.

Clonogenic assays of leukemic cells. Assays for the clonogenic potential of the leukemic cell lines were carried out as previously described (7). Briefly, 500 cells (or 333 for K562) in RPMI-C were plated in triplicate in alpha MEM medium containing 0.8% methylcellulose, 30% BCS, 0.3% bovine serum albumin, 5×10^{-5}M mercaptoethanol and 5.6×10^{-8}M selenium. The plates were incubated in moist 5% CO_2 at 37°C for 7–14 d and colonies (>40 cells) scored.

Clonogenic assays for NMPC. Progenitor cells were assayed in triplicate by a modification of the method of Fauser and Messner (28) as previously described (7). Briefly, culture conditions were similar to the clonogenic assay for leukemic cells with the exception that both phytohemagglutinin stimulated lymphocyte conditioned media (PHA-LCMK) and human placental conditioned medium and 1 unit/mL erythropoietin (Step III Connaught Laboratories, Willowdale, Ontario, Canada) were added. Later, NMPC assays were done with 1% methylcellulose in Iscove's modified Dulbecco's medium, 30% FBS, 5×10^{-5}M mercaptoethanol, 5% PHA-LCM and 2 units/mL Epotin Alpha (Amgen, Inc., Thousand Oaks, CA) with comparable results. The plates were scored on day 14. The total number of progenitors was defined as the sum of mixed colonies (GEMM), erythroid colonies (CFU-E), erythroid bursts (BFU-E), granulocyte-macrophage colonies (GM-CFU) (>40 cells) and GM clusters (<40 cells).

PKC assay. PKC activity was assayed as previously described (29,30). Briefly, partially purified PKC from pig brain or purified PKC from rat brain was used. The reaction mixture (0.2 mL) contained 5 μmol of piperazine-*N,N'-bis*(2-ethanesulfonic acid) (pH 6.5), 2 μmol $MgCl_2$, 5 μg of phosphatidylserine, 40 μg of histone H1, 0.06 μmol of ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N'N'*-tetraacetic acid, with or without 0.1 μmol of $CaCl_2$, 1.0 nmol of [γ-^{32}P]ATP (containing about 1×10^6 cpm) and varying concentrations of glycerophospholipid analogs dissolved in water by sonication. The reaction was carried out at 30°C for 5 min.

Statistical methods. The IC_{50} was defined as the concentration of compound which caused a 50% inhibition of the control value for any given assay. Repeated experiments were performed and the means used for comparisons of compounds using standard *t*-tests. Where the inhibition appeared linear, the IC_{50} was calculated using the least-squares method. Nonlinear inhibitions were graphed and IC_{50} determined by inspection. A therapeutic index was calculated by dividing the IC_{50} for NMPC by the IC_{50} for the clonogenic leukemic cells.

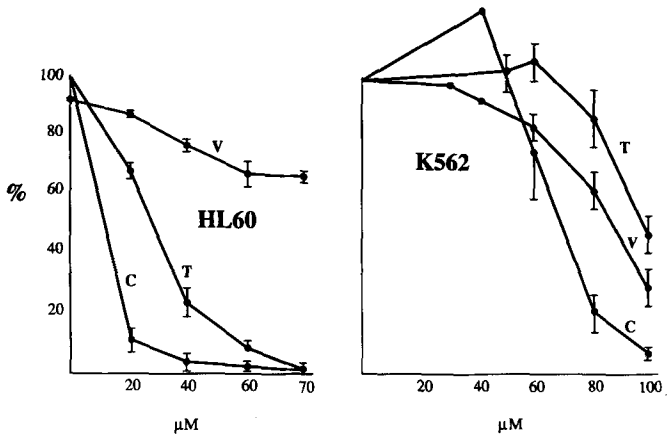


FIG. 2. Effect of a 4 h incubation of ET-18-OCH_3 on colony formation, [^{3}H]thymidine incorporation and viability in HL60 and K562 cells. Colony formation (C) and [^{3}H]thymidine (T) incorporation are expressed as percent of controls. The plating efficiency of K562 cells varies between 36 and 49% in controls, and in HL60 controls vary 13–36%. Average cpm uptake of T for 4×10^5 control for K562 and HL60 cells is in excess of 10^4. Viability (V) is the percentage of cells excluding trypan blue. Bars are standard errors of means of a minimum of three separate experiments.

RESULTS

Figure 1 gives the structure of the compounds investigated. In the *sn*-1 position, variations in chain length (16 or 18 carbons) and the substitution of sulfur for oxygen were the modifications tested. In the *sn*-2 position, a series of substitutions of varying composition, as indicated in Figure 1, were tested.

We observed two types of cytotoxicity. At high doses (>60 μM) cells were lysed, presumably because of a detergent effect of the phospholipids. Lower doses demonstrated no effect or, in some instances, an increase in colony formation and thymidine incorporation. The other type of cytotoxicity noted was a dose-related near linear reduction in colony formation and thymidine incorporation. These types of cytotoxicity are illustrated in Figure 2. In the HL60 cell line, which is sensitive to ET-18-OCH_3, there is a marked reduction in colony formation and [^{3}H]thymidine incorporation with no major change in cell viability. In contrast, in K562 cells which are relatively resistant to ET-18-OCH_3, there is an initial increase in colony formation and [^{3}H]thymidine incorporation at lower doses followed by a decrease at higher doses which parallels the decrease in the percentage of viable cells.

Table 1 gives the IC_{50} for each of the compounds with respect to colony formation for each of the cell lines tested. Because compound A has been the most widely studied and is active against a variety of human tumors (31), all of the others were compared to A. Except in the Daudi cell line, where compounds C and I were significantly more active than compound A, none were more active. On the other hand, several compounds were significantly less active than compound A. In HL60 cells, compounds C, D, K and N were less active and compound E, which has a long chain substitution at *sn*-2, was inactive. In K562, compounds G_2, I and K were inactive and compounds D and G_1 were less active than compound A. The IC_{50} values for NMPC were somewhat similar and did not vary significantly from compound A, except for compound G_1, which was more active.

Table 2 gives the data and the effects of the compounds upon DNA synthesis. Except for compound H in the Daudi cell line, none were significantly more active than compound A. In HL60, compounds C, K and N were significantly less active. In K562, compounds G_1, G_2, H, I, J and K had no inhibitory effect and compounds C and D were less effective. In Daudi, compounds C, G_1, J and K had low activity, whereas in U-937 compounds D, G_1, G_2, H, J and K were less active.

Table 3 shows the calculated therapeutic indices for the compounds obtained by dividing the IC_{50} for the NMPC by the IC_{50} for each of the cell lines. As can be seen, compounds A, H, I and J showed the highest values, indicat-

TABLE 1

Comparative Potency of Inhibition by Alkyl-lysophospholipid (ALP) of Clonogenicity of Various Cell Lines and Normal Marrow Progenitor Cells (NMPC)[a]

Compound	IC_{50}				
	HL60	K562	Daudi	U-937	NMPC
A	(4) 24.5 ± 2.3	(5) 71.6 ± 8.6	(5) 79.6 ± 7.87	(6) 52.8 ± 6.0	(13) 142.2 ± 11.6
B	(2) 29.9 ± 0.9	(3) 129.0 ± 28.6	(3) 53.2 ± 0.55	(4) 65.8 ± 14.7[d]	(4) 117.3 ± 10.2
C	(5) 41.7 ± 2.1[b]	(5) 204.0 ± 71.2	(3) 46.9 ± 2.25[d]	(4) 64.6 ± 5.5[d]	(2) 96.6 ± 13.4
D	(2) 51.1 ± 0.1[b]	(5) 129.7 ± 21.7[d]	(3) 65.8 ± 7.40	(2) 48.0 ± 2.1	(2) 99.4 ± 5.4
E	(2) NI[e]				
G_1	(3) 27.8 ± 3.2	(3) 410.1 ± 22.8[b]	(2) 125.5 ± 13.5[d]	(2) 42.7 ± 0.4	(2) 66.9 ± 3.5[d]
G_2	(3) 33.6 ± 4.0	(1) NI	(2) 151.1 ± 19.2[c]	(2) 44.5 ± 0.5	(1) 94.7
H	(8) 28.4 ± 3.3	(2) 71.0 ± 8.0	(1) 76.4	(3) 132.6 ± 7.8[b]	(2) 173.2 ± 9.5
I	(4) 19.4 ± 1.3	(3) NI	(2) 42.0 ± 1.5[d]	(3) 63.8 ± 3.5	(3) 116.2 ± 9.2
J	(5) 30.1 ± 3.3	(2) 104.9 ± 13.6	(4) 133.4 ± 27.8	(2) 47.8 ± 2.5	(3) 153.2 ± 24.2
K	(2) 44.7 ± 6.7[d]	(2) NI	(4) 152.9 ± 30.5[d]	(2) 43.3 ± 0.3	(3) 141.5 ± 40.2
L	(2) 29.3 ± 2.9	(2) 55.1 ± 3.8	(2) 75.6 ± 19.5	(6) 86.0 ± 45.6	(4) 104.6 ± 19.0
N	(3) 102.6 ± 1.8[b]				

[a]The data presented are means ± SE of 1–13 experiments (numbers in parentheses) done in triplicate. ALP exposure was 4 h.
[b]$P < 0.0005$.
[c]$P < 0.01$.
[d]$P < 0.05$.
[e]NI, no inhibition up to 500 μM.

TABLE 2

Comparative Potency of Inhibition by Alkyl-lysophospholipid (ALP) of Thymidine Uptake in Various Cell Lines[a]

Compound	IC_{50} (μM)			
	HL60	K562	Daudi	U-937
A	(9) 34.9 ± 3.2	(5) 108.3 ± 5.0	(5) 66.9 ± 0.0	(8) 51.5 ± 3.3
B	(3) 33.8 ± 0.3	(3) 139.7 ± 17.3	(3) 64.3 ± .9	(4) 58.4 ± 3.9
C	(4) 53.0 ± 6.2[d]	(2) 343.2 ± 63.7[b]	(3) 116.0 ± 3.5[b]	(4) 78.9 ± 19.1
D	(1) 43.3	(2) 305.1 ± 81.5[c]	(3) 95.9 ± 20.2	(2) 91.4 ± 2.1[b]
E	(2) NI[e]	—	—	—
G_1	(3) 45.8 ± 2.2	(1) NI	(2) 90.0 ± 7.0[d]	(2) 70.2 ± 0.6[d]
G_2	(2) 51.7 ± 3.0	(1) NI	(2) 81.7 ± 6.3	(2) 76.0 ± 2.1[d]
H	(7) 34.7 ± 5.4	(3) NI	(2) 47.7 ± 2.9[d]	(3) 91.9 ± 2.8[b]
I	(5) 41.7 ± 14.0	(3) NI	(3) 104.5 ± 37.6	(3) 61.2 ± 1.7
J	(5) 46.1 ± 3.8	(2) NI	(3) 142.6 ± 9.8[b]	(2) 89.5 ± 6.7[c]
K	(3) 77.2 ± 22.9[c]	(2) NI	(3) 165.1 ± 44.4[d]	(2) 72.0 ± 0.7[d]
L	(2) 33.9 ± 4.69	(2) 78.7 ± 11.9	(2) 47.8 ± 6.1	(6) 60.0 ± 2.7
N	(3) 106.7 ± 3.2[b]	—	—	—

[a]The data presented are means ± SE of 1-8 experiments (numbers in parentheses) done in triplicate. ALP exposure was 4 h.
[b]$P < 0.005$.
[c]$P < 0.01$.
[d]$P < 0.05$.
[e]NI, no inhibition up to 500 μM.

TABLE 3

Comparative Therapeutic Index of Alkyl-lysophospholipid[a]

Compound	Therapeutic index			
	HL60	K562	Daudi	U-937
A	5.80	1.99	1.79	3.32
B	3.92	0.91	2.20	1.78
C	2.32	0.47	2.06	1.50
D	1.95	0.77	1.51	2.07
G_1	1.60	0.16	.53	1.57
G_2	2.82		.63	2.13
H	6.10	2.44	2.27	1.31
I	6.00		2.77	1.82
J	5.09	1.46	1.15	3.21
K	3.17		0.93	3.27
L	3.57	1.90	1.38	1.22

[a]The index was calculated as described in Materials and Methods.

TABLE 4

Comparative Potency of Inhibition of Protein Kinase C by Alkyl-lysophospholipid[a]

Compound	IC_{50} μM
A (ET-18-OCH_3)	9
B	6
C	24
D	23
E	NI[b]
G1	NI
G2	NI
H	5
I	16
J	14
K	47
L (BM 41.440)	9
N	60

[a]The data presented are means of two experiments with experimental errors being less than ±5%. ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine.
[b]NI, no inhibition up to 500 μM.

ing a higher degree of selectivity. Table 4 gives the IC_{50} values for the various compounds on PKC activity. Compounds A, B, H and L proved to be the most active inhibitors.

DISCUSSION

Various substitutions at the *sn*-1 and *sn*-2 positions of glycerophosphocholine were tested to determine what structural changes influenced the selective antitumor activity of these ether lipids. The *sn*-3 substitutions were identical. All of the compounds had either a 16 or 18 carbon chain at *sn*-1. This difference in chain length appeared to make no difference (*i.e.*, compare compounds A and I as well as B and H) as long as the substitution at *sn*-2 was a methoxy group. Substitution of a sulfur for an oxygen linkage at *sn*-1 resulted in less activity (compare compounds B and K) when the *sn*-2 substitution was an acetamide. However, this did not prove to be true when the substitution at *sn*-2 was a methoxymethyl group (compare compounds K and L). In fact, compound L was quite similar to compound A in cytotoxicity.

sn-2 Substitution had a greater impact on activity. A long chain substitution (compound E) at *sn*-2 eliminated any cytotoxic activity and had no effect on PKC. The introduction of a trifluoroacetyl group in compound N (which is less hydrophobic than the acetyl group in compound H) reduced the activity significantly. The methoxy substitution at *sn*-2 was somewhat more cytotoxic than the thiomethyl moiety (compare compounds I and J) although the PKC inhibitory activity was similar.

The chiral *sn*-2 deoxy analogs (compounds G_1 and G_2) had similar activities, although substantially lower than those exhibited by compound A, and appeared to be more toxic to the NMPC. They had the lowest therapeutic index and were inactive toward PKC.

Other substitutions at *sn*-2 did not enhance cytotoxicity over that observed in compound A. The methoxycarbonyl-

amino substitution (compound C) had an effect similar to that of the methyl carbonyl substitution (compound B). Introducing a methanesulfonamide moiety (compound D) which adds an additional oxygen did not increase potency (compound B).

These ALP analogs differ from the naturally occurring acetyl derivative, platelet-activating factors (PAF), in that they have antitumor activity, but have little or no PAF-like activity (32,33). Lai *et al.* (34) synthesized a spin label derivative of ET-18-OCH_3. They also synthesized a derivative of PAF (an ester linkage at *sn*-2) and another compound with ester linkages at *sn*-1 and *sn*-2. Neither inhibited thymidine incorporation. Electron spin resonance indicated that the ether chains were less mobile than the ester chains. Also, the former, being more hydrophobic, appeared to promote packing of the two *O*-alkyl chains.

It has been suggested that the cytotoxicity of ALP may result from ALP's combined effects on certain membrane targets (such as PKC, Na, K-ATPase, Na pump and certain phospholipid-metabolizing enzymes) whose synergism may result in cell damage or death (35). The effects of ALP analogs on cells (Tables 1 and 2) are likely to be specific and do not seem to be due to a general membrane perturbation brought about by the ALPs. We reported previously that compound A (ET-18-OCH_3) inhibited TPA-induced differentiation (a PKC-mediated process) and ^{86}Rb uptake (an indicator of Na pump activity) of HL60 cells, both with an IC_{50} of 9 μM (35), a value identical to that for its inhibition of PKC (35; also see Table 4). Taking into consideration factors such as binding of ALP to serum in the medium and uptake of the agent into the cell membrane, the ratio of the agent to total membrane phospholipid was estimated to be about 0.036 mol%, assuming that 7×10^5 cells contained 1,250 nmol of membrane phospholipids (35). Chabot *et al.* (36) and Diomede *et al.* (37) have determined the phospholipid content to be 9.1 and 27.0 nmol, respectively, for the same number of cells. The ratios of compound A to total membrane phospholipid, accordingly, were much higher (4.9 and 1.7 mol%, respectively) when their values for phospholipid were used. The IC_{50} values of compound A for clonogenicity (Table 1) and thymidine uptake (Table 2) for various leukemia cell lines and NMPC from the present studies ranged from 24.5 to 142.2 μM. The mol% of the agent to total cell membrane phospholipid was estimated accordingly, to be about from 13.3 to 26.9, respectively, using the phospholipid content reported by Chabot *et al.* (36). It is thus conceivable that incorporation of compound A into a biomembrane will have a profound effect on a number of enzymes, including PKC, which could contribute to the cytotoxicity of the ether lipids.

Additional studies of ALP derivatives will be necessary to further assess the structural requirements of antitumor active PAF analogs. Nevertheless, the present series of tumor-cytotoxicity studies and determination of therapeutic indices in comparison of healthy bone marrow progenitor cells provide a number of ueful leads toward the design and development of more potent antitumor active phospholipids. Demonstrating selective and specific tumor cytotoxicity of either 16 or 18 carbon chain *sn*-1-alkyl ether and thioether analogs containing short-chain *sn*-2-alkyl- or acetamido-deoxy- as well as an alkoxymethyl-substituted phospholipid confirms and expands the earlier attempts to delineate structural requirements of antitumor active phospholipids and provides an additional set of criteria (38). The functional role of the *sn*-3-phosphodiester substitution can now be addressed further, as it has been shown that PAF analogs containing headgroup bases other than choline exhibited significant antitumor activity (39).

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Effect of Age and α-Linolenic Acid Deficiency on Δ6 Desaturase Activity and Liver Lipids in Rats

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The combined effects of age and of diet deficient in n-3 fatty acids on Δ6 desaturation of linoleic acid and on lipid fatty acid composition were studied in the liver of the rat at 2, 6, 12, 18 and 24 mon of age. The profiles of Δ6 desaturase activity and fatty acid composition were studied in the deficient rats refed, at these different ages, either with 18:3n-3 (mixture of peanut and rapeseed oils) or with 20:5n-3 + 22:6n-3 (fish oil) diets for 2, 4, 8 or 12 wk. Results showed that the liver Δ6 desaturation activity in the control rats remained high at 2 and 6 mon, decreased by 30% from 6 to 12 mon, and then remained stable from 12 to 24 mon. In the deficient rats, this activity remained high during the entire period studied. Thus, the profile of liver Δ6 desaturase activity after puberty was not related to age only; it also depended on the polyunsaturated fatty acid (PUFA) n-6 and n-3 balance in the diet. In the controls, in parallel with the Δ6 desaturase activity, PUFA metabolism could be divided into three periods: a "young" period, and "old age" period, separated by a period of transition between 6 and 12 mon. Recovery from PUFA n-3 deficiency occurred at all ages but in a different manner depending on whether the rats were "young" or "old." Recovery was faster if long-chain n-3 PUFA rather than α-linolenic acid were supplied in the diet.

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Some long-chain polyunsaturated fatty acids (PUFA) of tissue and serum lipids are supplied directly by the diet, but most are derived by desaturation and elongation of their precursors, linoleic and α-linolenic acids. The first step in this process is catalyzed by a microsomal enzyme, Δ6 desaturase. The two precursor fatty acids compete for this enzyme (1,2); 18:3n-3 is desaturated at a higher rate than 18:2n-6. In the presence of the two dietary fatty acids and at equal concentrations, 18:3n-3 effectively inhibits the desaturation of 18:2n-6 (3). Δ6 Desaturase activity is also inhibited by the products of the desaturation-elongation process due to feedback inhibition by 20- and 22-carbon fatty acids (4,5). Studies on rats after puberty have shown that age affects Δ6 desaturase activity (6–8). Some investigators showed that this activity decreased progressively with age (6,7), others that it fluctuated (8). These variations in enzyme activity were reported to be either accompanied by changes in tissue lipid composition (6,7), or not (8). Other authors have studied the fatty acid profile in the liver of rats ranging in age from 1.5 to 40 mon; only the proportion of 20:4n-6 was shown to decrease with age (9). When animals given an α-linolenic acid-deficient diet were refed a balanced PUFA diet, the composition of tissue phospholipids returned to normal within variable times depending on the organ studied. However, these studies were carried out only on young animals, and the n-3 PUFA used in refeeding was derived from vegetable oil (10,11).

The aim of the present work was to study (i) the combined effects of age and dietary n-3 PUFA deficiency on changes in the activity of Δ6 desaturase on 18:2n-6 and changes in the fatty acid composition of liver total lipids in male rats between 2 and 24 mon of age, and (ii) the combined effects of age and of refeeding with α-linolenic acid or its long-chain derivatives on the two parameters.

MATERIALS AND METHODS

Animals and diets. Two generations of female Wistar rats were given a diet containing lipids in the form of African peanut oil (5%, w/w) low in α-linolenic acid; this diet (deficient) supplied about 900 mg of linoleic acid but only 5 mg of α-linolenic acid/100 g of diet. Two weeks before mating, the deficient second-generation females were divided into two groups. The first group continued to receive the deficient diet while the second group was given a diet in which the peanut oil was replaced by a mixture of peanut and rapeseed oils (50:50). This diet (control) supplied the same amount of linoleic acid as the deficient diet but also about 200 mg of α-linolenic acid/100 g of diet (n-6/n-3 = 5). At weaning, the male progeny of these two female groups were given the same diet as their respective dam, and animals were killed when 2, 6, 12, 18 or 24 mon old. At each of these ages some deficient animals were refed with n-3 PUFA, one-half with the control diet described above and the other half with a diet containing a mixture of peanut oil and cod liver oil (92.5–7.2%, respectively); thus, the first half was refed with n-3 PUFA with α-linolenic acid and the other with a mixture of eicosapentaenoic and docosahexaenoic acids (EPA plus DHA). The latter diet supplied about 900 mg of n-6 PUFA (like the other diets) and 100 mg of long-chain n-3 PUFA/100 g of diet. This amount of long-chain n-3 PUFA was chosen because of a preliminary study had shown that it was equivalent to 200 mg of α-linolenic acid, which is sufficient to satisfy the n-3 PUFA requirement (11). Some animals were killed at 2, 4, 8 or 12 wk (T2, T4, T8 and T12) after refeeding with n-3 PUFA. The composition of the diets and the fatty acid composition of the lipids are given in Tables 1 and 2.

Measurements of Δ6 desaturase activity. Non-fasted animals were killed between 8:00 and 9:00 a.m. to ensure optimal conditions for Δ6 desaturase measurement (12) and to avoid any variation in Δ6 desaturase activity due to circadian rhythm (13). The liver was rapidly excised and weighed. One part was used to measure enzyme activity and the rest for fatty acid analysis.

Specific Δ6 desaturase activity was assayed using the method of Blond and Lemarchal (14) as modified by Bourre *et al.* (15). The liver was homogenized at 4°C in 0.25 M saccharose buffer containing 0.05 M Na_2HPO_4, 2 mM glutathione, pH 7.4 (5 mL of buffer/2 g fresh tissue). The homogenate was centrifuged at 12,000 × *g* for 15 min

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TFA, total fatty acids.

TABLE 1

Diet Composition (g/kg)

Constituents	g/kg
Casein	220.0
DL Methionine	1.6
Mineral mixture[a]	40.0
Vitamin mixture[b]	10.0
Cellulose	20.0
Oil[c]	50.0
Starch	439.0
Saccharose	219.4

[a]Composition (g/100 g):$CaHPO_4 \cdot 2H_2O$, 38.0; K_2HPO_4, 24.0; $CaCO_3$, 18.0; NaCl, 6.9; MgO, 2.0; $MgSO_4 \cdot 7H_2O$, 9.0; $FeSO_4 \cdot 7H_2O$, 0.86; $ZnSO_4 \cdot H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.1; NaF, 0.08; $CrK(SO_4)_2 \cdot H_2O$, 0.05; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.002; KI, 0.004; $CoCO_3$, 0.002; $Na_2SeO_3 \cdot 5H_2O$, 0.002.

[b]Total vitamin supplement, United States Biochemical Corp. (Cleveland, OH).

[c]Control diet consists of a mixture of peanut oil and rapeseed oil (50:50), deficient diet consists of peanut oil, refeeding diets consist of a mixture of peanut oil and rapeseed oil (50:50) or a mixture of peanut oil and cod liver oil (92.5:7.5).

to precipitate cell debris, mitochondria and nuclei. The supernatant contained both microsomes and cytosol. The latter increased the activity of acyl-CoA synthetase activity, thus favoring the formation of acyl-CoA (14) serving as substrates of desaturases. The supernatant was diluted twice with the same buffer before the protein content was determined by the method of Lowry *et al.* (16). Five mg of protein and 100 nmol of [1-^{14}C]18:2n-6 (2 μCi, 20 μL) were added. A final volume of 2 mL was obtained using the same buffer as before containing the following cofactors: Na_2HPO_4 (50 mM) ATP (7.5 mM), $MgCl_2$ (3.8 mM), NADPH (0.2 mM), NADH (0.5 mM) and CoA (0.2 mM). The tubes were incubated for 30 min with shaking. The reaction was stopped by the addition of 1 mL of KOH in 12% ethanol. Thirty μg each of several standard commercial fatty acids (18:2n-6, 18:3n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:6n-3) were added to facilitate identification of chromatographic fractions. After saponification at 100°C for 30 min, 0.4 mL of 10 N HCl was added, the fatty acids were extracted using hexane, followed by methylation with 1 mL of 14% boron trifluoride for 1 h at 100°C. The fatty acid methyl esters were extracted with hexane, purified by thin-layer chromatography using a mixture of petroleum ether/diethyl ether (80:20, vol/vol), and then were localized by autoradiography. The methyl ester band was scraped from the plate and extracted successively with hexane (2 times 3 mL) and diethyl ether (2 times 3 mL). The methyl esters were finally separated according to their degree of unsaturation by $AgNO_3$ thin-layer chromatography. Plates were impregnated with 10% $AgNO_3$ in acetonitrile for 15 min, then dried at 100°C for 20 min.

TABLE 2

Fatty Acid Composition of Dietary Lipids[a]

	Diets		
	Peanut oil	Peanut oil (50%) Rapeseed oil (50%)	Peanut oil (92.5%) Cod liver oil (7.5%)
Fatty acids (%)			
14:0	0.4	0.5	0.9
16:0	11.9	10.1	10.9
18:0	2.9	2.3	3.3
20:0	1.2	0.8	1.3
22:0	1.9	1.1	2.3
24:0	0.9	0.6	1.0
Total SFA	19.2	15.4	19.7
16:1n-7	0.6	0.7	1.6
18:1n-7	3.1	6.0	2.9
18:1n-9	56.1	53.3	52.3
20:1n-9	0.9	0.8	1.7
22:1n-11	—	—	0.8
Total MUFA	60.7	60.8	59.3
18:2n-6	19.9	19.8	18.5
20:4n-6	—	—	0.2
Total n-6 PUFA	19.9	19.8	18.7
18:3n-3	0.1	3.7	0.3
20:5n-3	—	—	0.8
22:5n-3	—	—	0.3
22:6n-3	—	—	0.9
Total n-3 PUFA	0.1	3.7	2.3
(n-6) + (n-3)	20.0	23.5	21.0
n-6/n-3	199.0	5.3	8.1
Total n-6 PUFA (mg/100/g of diet)	935.0	931.0	879.0
Total n-3 PUFA (mg/100/g of diet)	5.0	173.9	108.0

[a]Abbreviations: SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

The mobile phase was a mixture of petroleum ether/diethyl ether (50:50, vol/vol). The fractions were localized by autoradiography, and the radioactive distribution was determined using a plate reader (Automatic TLC Linear Analyzer Berthold, La Garenne Colombes, France). Results were expressed in pmol of 18:3n-6 formed/mg protein/min incubation.

Fatty acid composition of liver total lipids. Liver lipids were extracted from frozen dried liver with a chloroform/methanol mixture (2:1, vol/vol) using the method of Folch *et al.* (17) as modified by Pollet *et al.* (18). The lipid extracts were then transmethylated (19). After extraction with hexane, the methyl esters were analyzed by gas chromatography, using a Carlo Erba (Rueil Malmaison, France) chromatograph with an automatic on-column injector, a flame-ionization detector and capillary-type Carbowax C.P. Wax 52 C.B. column. Data were processed using a Stang microcomputer (Pavillon sous Bois, France) and Nelson software (Cupertino, CA).

Analysis of results. This study required a large number of animals: 144 male rats were killed at various ages. Nevertheless, only a small number of animals could be studied at each time point (3 or 2 rats). Liver total lipid fatty acid composition was analyzed for only two rats per time period; thus common statistical procedures could not be applied. High mortality in the groups of rats 24 mon of age or older did not permit measurements at 12 wk of refeeding.

RESULTS

Effect of age and dietary α-linolenic acid deficiency. In control rats, the specific Δ6 desaturase activity (Fig. 1) remained constant at 2 and 6 mon; it decreased by about 30% between 6 and 12 mon and then did not change between 12 and 24 mon. Enzyme activity in α-linolenic acid-deficient animals was comparable to that in controls at 2 and 6 mon, but instead of decreasing thereafter it remained high during the entire period studied; thus, from 12 to 24 mon, enzyme activity was 50% higher than in controls.

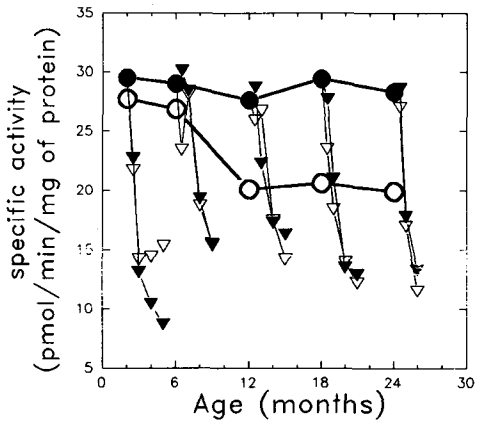

FIG. 1. Effect of age and α-linolenic acid deficiency on Δ6 desaturase activity in the rat. Effect of refeeding n-3 polyunsaturated fatty acids (PUFA) according to age and nature of n-3 PUFA (α-linolenic acid or long-chain n-3 PUFA). Assays of Δ6 desaturase were carried out as described in Materials and Methods; ○, control rats; ●, deficient rats; ▽, 18:3n-3 refed rats; ▼, 20:5n-3 + 22:6n-3 refed rats. Values are the mean of three or two rats/diet.

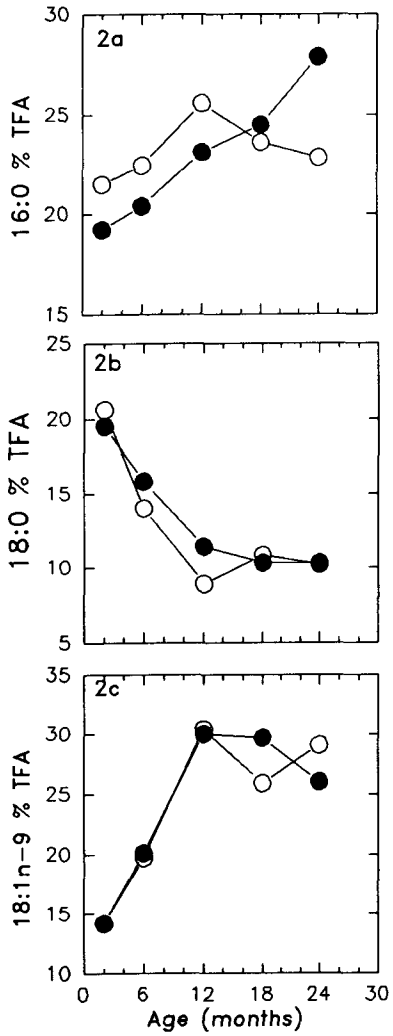

FIG. 2. Effect of age and α-linolenic acid deficiency on levels of the main saturated fatty acids and monounsaturated fatty acids of liver total lipids in the rat. Only values for the major fatty acids are reported. Experimental details are given in Materials and Methods. Data were derived from the percent of total fatty acids (TFA); ○, control rats; ●, deficient rats. Values are the mean of two rats/diet.

Fatty acid composition was also affected by the diet. Palmitic (16:0) and stearic (18:0) acids were the major saturated fatty acids (SFA). In controls, the proportion of palmitic acid was unchanged (21 to 23%) between 2 and 24 mon (Fig. 2a), but stearic acid levels decreased by half between 2 and 12 mon and then remained constant up to 24 mon (Fig. 2b). The 18:0 profile affected the proportion of total SFA which decreased from 44 to 36% between 2 and 12 mon and then remained stable until 24 mon (Table 3). Generally, an n-3 PUFA deficient diet had very little influence on the proportions of liver lipid SFA and monounsaturated fatty acids (MUFA). However, in the deficient rats, 18:0 decreased with age, as in the controls, and 16:0 increased regularly so that, as the decrease of one compensated the increase of the other, the level of total SFA remained almost constant from 2 to 24 mon (between 36 and 40% of total fatty acids). The MUFA time course was similar in control and deficient animals: their proportions doubled between 2 and 12 mon (from 20 to 40%) at

TABLE 3

Effect of Age and of n-3 PUFA Deficiency on Fatty Acid Composition of Liver Total Lipids[a]

	Diet									
	C (2)[b]	D (2)	C (6)	D (6)	C (12)	D (12)	C (18)	D (18)	C (24)	D (24)
Fatty acids (%)										
Total SFA	43.7	39.8	38.1	38.2	36.0	35.9	36.2	36.7	34.3	40.1
Total MUFA	20.5	20.7	28.4	28.8	41.4	39.1	36.4	39.3	38.7	36.1
n-6 ⩾ 20 C	18.8	29.0	17.2	23.5	11.4	15.8	11.0	14.2	11.1	14.4
Total n-6 PUFA	29.9	38.6	26.3	28.8	22.6	24.1	22.8	23.3	22.5	22.7
n-3 ⩾ 20 C	5.8	1.1	6.8	1.1	3.9	0.8	4.2	0.7	4.0	0.8
Total n-3 PUFA	6.2	1.1	7.1	1.1	4.1	0.8	4.7	0.7	4.5	0.8
n-6 + n-3 ⩾ 20 C	24.6	30.1	24.0	24.6	15.3	16.6	15.2	14.9	15.1	15.2
Total (n-6) + (n-3)	36.1	39.7	33.4	33.0	26.7	24.8	27.4	24.0	27.0	23.5
n-6/n-3	4.8	35.1	3.7	29.0	5.5	32.1	4.9	33.0	5.1	28.4
22:5n-6/22:6n-3	0.04	3.70	0.04	3.40	0.05	2.40	0.03	1.60	0.03	1.30
20:4n-6/18:2n-6	1.81	2.82	2.03	2.72	1.08	1.79	1.00	1.58	1.00	1.79

[a]The values are the mean of two or three rats/diet. C, control rats; D, deficient rats; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]Number in parentheses is age in months.

the same time as oleic acid increased from 14 to 28% (Fig. 2c); between 12 and 24 mon there was no change.

As expected, the n-6 PUFA profiles were different in control and deficient animals: in the former the proportion of total n-6 PUFA decreased from 30 to 23% between 2 and 12 mon and then stabilized. This overall relatively moderate decrease was due entirely to arachidonic acid (20:4n-6) which decreased from 18 to 11% between 2 and 12 mon (Fig. 3b), while the proportion of linoleic acid was constant (about 10%) (Fig. 3a). In deficient animals, the proportion of 18:2n-6 (which did not vary with age) was systematically lower than in controls. On the other hand, 20:4n-6 and especially 22:5n-6 levels were higher (Fig. 3c). However, the level of 22:5n-6, higher from 2 to 6 mon, diminished considerably between 6 and 12 mon. After 12 mon, the higher values of 20:4n-6 and 22:5n-6 just compensated for the lower value of 18:2n-6, so that total n-6 PUFA became similar in control and deficient rats after 12 mon (about 23%). The level of 20:3n-6 did not vary with either age or diet. DHA (22:6n-3) was the main n-3 PUFA in controls. It represented 5–6% of n-3 PUFA up to 6 mon, dropped between 6 and 12 mon to about 3.4% and then remained at that level until 24 mon (Fig. 3d). The total n-3 PUFA profile was comparable. In deficient rats, n-3 PUFA were represented only by 22:6n-3, and it proportion remained at about 1% during the whole period studied. Total n-6 + n-3 PUFA in controls remained at the same level from 2 to 6 mon, decreased by about 20% between 6 and 12 mon and then stabilized; in deficient animals, the same pattern was seen but the plateau reached at 12 mon was lower due to a lower proportion of n-3 PUFA which was no longer compensated for by a high n-6 PUFA level (Fig. 3e). If only long-chain PUFA (⩾20 C) are considered instead of total PUFA, there was overcompensation for the n-3 PUFA deficiency by n-6 PUFA at 2 mon but not at other time points. In controls, the n-6/n-3 ratio remained about 5 throughout the period studied; it was 6 to 7 times higher in deficient rats (Fig. 3f). The 22:5n-6/22:6n-3 ratio, which may be considered an index of dietary n-3 PUFA deficiency (20), did not change in controls. It was high in deficient rats but decreased progressively with age. In controls the 20:4n-6/18:2n-6 ratio, an index of total Δ6 and Δ5 desaturase activities (21), remained high and stable from 2 to 6 mon, dropped by one-half between 6 and 12 mon and then remained stable until 24 mon. The profile of the deficient rats was comparable but the ratio was higher.

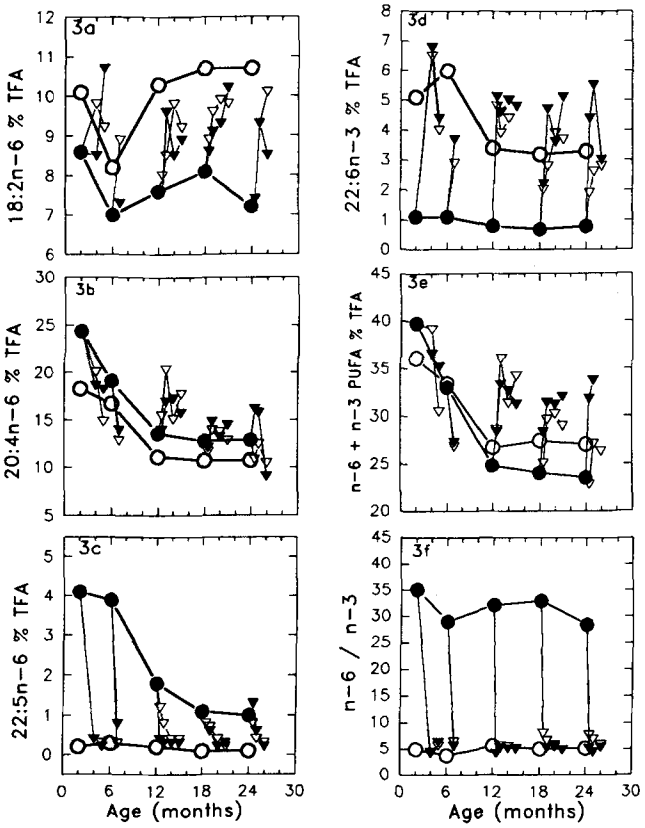

FIG. 3. Effect of age and α-linolenic acid deficiency on the levels of the main polyunsaturated fatty acids (PUFA) of liver total lipids in the rat. Effect of refeeding n-3 PUFA according to age and nature of n-3 PUFA (α-linolenic acid or long-chain n-3 PUFA). Experimental details are given in Materials and Methods. Data were derived from the percent of total fatty acids (TFA); ○, control rats; ●, deficient rats; ▽, 18:3n-3 refed rats; ▼, 20:5n-3 + 22:6n-3 refed rats. Values are the mean of two rats/diet.

Effect of PUFA n-3 refeeding in deficient rats. Generally, the addition of n-3 PUFA to the diet, whether in the form of 18:3n-3 or of 20:5n-3 + 22:6n-3, caused the Δ6 desaturase activity to drop by one-half to a level lower than that in controls of the same age (Fig. 1). The inhibition appeared much sooner in 2-month-old animals, independent of the nature of the PUFA n-3 in the diet. In older animals there was a latency period of about 2 wk before the inhibition became evident, especially when the rats received long-chain n-3 PUFA. Maximal inhibition was obtained only after 8 wk of refeeding.

The addition of 18:3n-3 to the diet of deficient rats modified the fatty acid composition of liver total lipid with a decrease in n-6 PUFA level between 2 and 6 mon. At these two ages, the values obtained at T8 for 2-month-old rats and at T4 for 6-month-old ones were very close to those of controls. This decrease was accompanied by a new balance between the 18:2n-6 on one hand and the 20:4n-6 and the 22:5n-6 fatty acids on the other. At the end of the first two wk of refeeding (T2), the level of 18:2n-6 increased and that of 22:5n-6 decreased; thus, there was a return to normal values (Figs. 3a,3c). The level of 20:4n-6 decreased to a level lower than that of controls (Fig. 3b). There were no differences between the levels of total n-6 PUFA in 12-, 18- and 24-month-old controls and deficient animals, and refeeding α-linolenic acid did not cause an overall change. However, refeeding did promote an increase in the level of 18:2n-6, which reached a plateau at the end of 4 wk but did not return to normal values. The levels of 20:4n-6 returned to normal only at 24 mon; it was practically stable at 18 mon and increased at 12 mon. The level of 22:5n-6 returned to normal values, but only after 8 wk of refeeding. The addition of 18:3n-3 to the diet of n-3 PUFA deficient animals caused an increase in liver 22:6n-3 (Fig. 3d). With the exception of 24-month-old rats, all the age groups returned to control values and even exceeded them. In 6-month-old rats, the values reached after 4 wk of refeeding showed that these rats were recovering, whereas in 24-month-old animals the level of 22:6n-3 remained lower than that in controls of the same age. Due to variations in the levels of different PUFA, total (n-6 + n-3) PUFA decreased in rats refed 18:3n-3 at 2 and 6 mon (Fig. 3e) because n-6 PUFA decreased more than n-3 PUFA increased. However, total PUFA were appreciably higher when rats were refed at 12 mon; this overcompensation disappeared with age, and at 2 yr total (n-6 + n-3) PUFA was the same as in controls. Refeeding 18:3n-3 returned the n-6/n-3 ratio and the 22:5n-6/22:6n-3 ratio to normal values within 4 wk (5 and 0.05, respectively) (Fig. 3f).

Refeeding fish oil had almost the same effect on liver n-6 PUFA as refeeding 18:3n-3. After refeeding long-chain n-3 PUFA, 18:3n-3 was not detected in liver lipids; 20:5n-3 and 22:5n-3 levels were lower than or equal to 0.5% (results not shown). Refeeding with 100 mg of 20:5n-3 + 22:6n-3/100 g of diet resulted in an increase in 22:6n-3 comparable to a supply of 200 mg of 18:3n-3/100 g of diet in 2-, 6- and 12-month-old rats. But in rats of 18 and 24 mon of age, the fish oil diet raised the level of 22:6n-3 more quickly than the rapeseed oil diet. After 2 wk of refeeding, the level was much higher than that seen in control rats of the same age. After this increase the values stabilized. In 12- and 18-month-old rats, the values remained higher than normal but in 24-month animals they returned to control values as observed after 8 wk of refeeding.

DISCUSSION

Effect of age. We found a decrease of 18:0 between 2 and 12 mon and an increase of 18:1n-9 during the same period, corroborating the results of Ulmann *et al.* (8) who found a slight decrease in the sum of 16:0 + 18:0 together with an increase of 16:1n-7 + 18:1n-9 in the liver microsomal phospholipids of rats 3-, 6- and 9-months old. In regard to the PUFA composition of liver total lipids, our study showed that the postpuberty period can be divided into three parts: (i) a period of stability between 2 and 6 mon; (ii) a transition period between 6 and 12 mon during which levels of 20:4n-6, 22:5n-6 and 22:6n-3 decrease; and (iii) another period of stability from 12 to 24 mon. Thus, the PUFA level was lower in old than in young rats. However, our results do not show whether the transition period extends over the whole 6 to 12 mon interval or whether it is shorter. Murawski *et al.* (9) also observed a decrease in the level of total liver lipid 20:4n-6 in rats receiving a standard diet and with ages ranging from 1.5 to 40 mon. But these authors found no variation in the proportions of other PUFA. In contrast to these results, Bordoni *et al.* (6) found that total liver lipid 20:4n-6 levels were not different in rats 4- and 22-months-old.

Our study shows that in animals receiving a balanced PUFA diet the variations in PUFA composition correspond to variations in specific Δ6 desaturase activity which, like total liver lipids, was similar at 2 and 6 mon, decreased between 6 and 12 mon and was stable thereafter. The 20:4n-6/18:2n-6 ratio also fell during this period. However, the 20:3n-6 level did not change. Thus, it seems that only Δ6 desaturase should be involved in the decrease of the 20:4n-6/18:2n-6 ratio; otherwise, we likely would have observed an increase in 20:3n-6 level if Δ5 desaturase was also involved (22). Peluffo and Brenner (23) showed that Δ6 desaturase activity tends to decrease with age in rats receiving a standard diet; but their experiment only concerned rats aged 3 mon and 1 yr and, therefore, any intermediate fluctuations would have been missed. Recently, Ulmann *et al.* (8) studying rats aged 3, 6 and 9 mon receiving a control diet similar to the ones in our study also showed that Δ6 desaturase activity decreased from 6 to 9 mon. However, other authors have not observed the same trend of Δ6 desaturase activity with age; Bordoni *et al.* (6) using liver microsomes of rats at 13 d and 1, 4, 14 and 22 mon which had received a standard diet noted that enzyme activity decreased progressively from 4 to 22 mon. In a similar study on rats aged 1, 6, 10 and 25 mon, Hrelia *et al.* (7) also found a linear decrease in Δ6 desaturase activity with age; in mice, Bourre *et al.* (15) noted that liver Δ6 desaturase activity did not change between 3 wks and 4 mon but progressively decreased between 4 and 17 mon. The discrepancy between the results of our study and those of Ulmann *et al.* (8) on one hand, and those of Bordoni *et al.* (6) and Hrelia *et al.* (7) on the other, could be due to different diet compositions since this, as we have shown, determines the profile of enzyme activity during postpubertal development.

Effect of dietary n-3 PUFA deficiency. The previous studies on dietary n-3 PUFA deficiency have shown that the deficiency causes an increase in 20:4n-6 and 22:4n-6 levels, and especially in 22:5n-6 levels, to compensate for the decrease in 18:2n-6, 22:5n-3 and especially 22:6n-3 (24–28). Other studies have shown that n-6 PUFA can

completely compensate for n-3 PUFA (22, 29–31) and that total PUFA (n-6 + n-3) remains constant. These studies on n-3 PUFA deficiency were done on 15-day-old and 60-day-old rats. Our results showed that this was not the case in animals from 12 mon of age, and that compensation was not adequate so that the sum of PUFA was lower in n-3 PUFA-deficient animals than in controls. However, if only fatty acids with at least 20 carbon atoms are considered, n-3 PUFA were exactly compensated for by n-6 PUFA. In other words, in the old, deficient animals, increases in 20:4n-6 and 22:5n-6 did not compensate for the decrease in 18:2n-6 but only for the decrease in 22:5n-3, and especially 22:6n-3. Moreover, in n-3 PUFA-deficient rats, results are remarkably consistent in regard to the lower level of 18:2n-6 and the higher level of 20:4n-6 and 22:5n-6 on one hand, and the higher level of Δ6 desaturase activity on the other, as compared to controls. In the deficient animals, specific enzyme activity did not change with age as it did in controls. Thus, it is evident that changes in Δ6 desaturase activity were not related solely to age but were also a function of diet. It is known that the desaturase activities, and especially the Δ6 desaturase activity, also depend on non-lipid dietary factors. These activities moreover depend on hormonal factors, the effects of which being obvious in some pathological conditions, such as diabetes. At last, if the influence of desaturases on the lipid composition of tissue lipids, and in particular that of microsomal phospholipids, is generally assumed, it should be observed that this composition also results from many other metabolic factors (for a review, see Ref. 32).

Effect of n-3 PUFA refeeding. Our results showed that, independent of the nature of dietary n-3 PUFA, the modalities of recovery in young animals (2- and 6-months-old) were clearly different from those in old animals (12-, 18- and 24-months-old). Refeeding tended to decrease the level of total liver lipid PUFA in the young, while this level was markedly increased in old animals so that it reached the level in young controls. Moreover, a supply of long-chain n-3 PUFA permitted a more rapid and more complete recovery than a supply of 18:3n-3, the difference being more evident in older animals. In this study, EPA plus DHA supplementation did not increase the level of 20:5n-3 to the detriment of 20:4n-6, as occurred when a larger amount of these fatty acids was supplied in the diet (33,34). It is likely that, because of the moderate supply of EPA plus DHA (100 mg/100 g of diet), 20:5n-3 was completely transformed into 22:6n-3 to cover the requirements for phospholipid 22:6n-3. The persistently low activity of Δ6 desaturase in refed rats compared to that in controls of the same age could be due to the fact that 22:5n-6 progressively released from phospholipids decreased Δ6 desaturase activity by feedback inhibition. This fatty acid may then be eliminated either by mitochondrial and/or peroxisomal oxidation, or by retroconversion into 20:4n-6. If in old rats the retroconversion is the usual pathway, this would explain why the high 20:4n-6 levels found in n-3 PUFA-deficient rats was maintained or even increased when the animals were refed with n-3 PUFA. This high 20:4n-6 level could in itself contribute to maintaining Δ6 desaturase activity at the low level seen after n-3 PUFA refeeding. In any case, Δ 6 desaturase activity at a given age may depend not only on the relative proportions of n-6 and n-3 PUFA in the current diet but also on the relative proportions in the diet eaten over a longer period of time.

In conclusion, this study shows for the first time that during adult rat life (puberty to old age), PUFA metabolism can be divided into three periods: two of stability ("young" and "old" ages), separated by a transition period. The latter occurs between 6 and 12 mon and its duration remains to be determined. In rats given a balanced PUFA diet, the transition period is characterized by a decrease in liver long-chain PUFA, in particular 22:6n-3 and 20:4n-6. Our results show that the profile of Δ6 desaturase activity as a function of age cannot be dissociated from the n-6 and n-3 PUFA ratio in dietary lipids. Thus, an n-3 PUFA-deficient diet maintains specific Δ6 desaturase activity at a high, constant level throughout adult life, from puberty to old age. Finally, it is possible to recover from multi-generational n-3 PUFA deficiency at all ages but the pattern of recovery is different, depending on whether the animal is in the "young" or the "old age" period. During the "old age" period, recovery is quicker if the diet directly supplies long-chain n-3 PUFA.

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Differential Utilization of Eicosapentaenoic Acid and Docosahexaenoic Acid in Human Plasma

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It has recently been shown that the ω3 fatty acid status in humans can be predicted by the concentration of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in plasma phospholipids [Bjerve, K.S., Brubakk, A.M., Fougner, K.J., Johnsen, H., Midjthell, K., and Vik, T. (1993) *Am. J. Clin. Nutr.*, in press]. In countries with low intake of ω3 fatty acids, the level of EPA in plasma phospholipids is often only about one-fifth the concentration of DHA. The purpose of this study was to investigate whether this difference in the concentration of these two fatty acids was due to a selective loss of EPA relative to DHA or to a lower dietary intake of EPA. Seven female volunteers ingested four grams of MaxEPA daily for 2 wk and in the following 4 wk they ate a diet almost completely devoid of the long-chain ω3 fatty acids. The concentrations of the ω3 fatty acids in the plasma cholesteryl esters, triglycerides and phospholipids and the high density lipoprotein phospholipids were examined at weekly intervals throughout the study. There was a more rapid rise in the concentration of EPA than in DHA levels in the supplementation period in all lipid fractions, but there was a disproportionate rise in DHA relative to EPA in the plasma lipids compared with the ratio in the supplement. In the depletion phase there was a rapid disappearance of EPA from all fractions, such that pre-trial levels were reached by one week post-supplementation. The disappearance of DHA was slower, particularly for the plasma phospholipids: at 4 wk post-supplementation, the DHA concentration in this fraction was still 40% above the pre-trial value. It is suggested that the low plasma EPA values relative to DHA are the result of increased β-oxidation of EPA and/or low dietary intake, rather than a rapid conversion of EPA to DHA. One practical result of this experiment is that, compared with DHA, the maintenance of increased EPA levels in plasma (and therefore tissues) would require constant inputs of EPA due to its more rapid loss from the plasma. *Lipids 28,* 525–531 (1993).

The ω3 fatty acids are now recognized as important components of the human diet. Following intense investigations during the last two decades, their role as modulators of the metabolism of arachidonic acid to eicosanoids (1), their importance in protecting against cardiovascular disease by a variety of actions (2) and their potential significance for the development of normal function of the retina and brain (3) has been established.

The main ω3 polyunsaturated fatty acids (PUFA) from the food chain are α-linolenic acid (from plant sources), eicosapentaenoic acid (20:5ω3 or EPA), docosapentaenoic acid (22:5ω3 or DPA) and docosahexaenoic acid (22:6ω3 or DHA). These latter three fatty acids, which can be found in high concentrations in marine foods, are often referred to as ω3 long-chain PUFAs (LCP). The interest in these PUFA was stimulated by the observations on the low incidence of cardiovascular disease in Greenland Eskimos despite the high fat content of their diet (4).

There is general agreement that the level of ω3 PUFA should be increased in the Western diet, and various suggestions have been made regarding an appropriate level of ω3 fatty acids in our current diet (5). In most Western countries the level of ω3 PUFA in the diet is low relative to the ω6 PUFA [estimated at 1:11 based on world production figures (6), and a similar ratio for the United States, based on consumption figures, (7)] and this is reflected in the balance of these fatty acids in plasma and certain tissues. The ratio of ω3 to ω6 PUFA in the diet in Australia is likely to be lower than that in the United States since many common vegetable oils used in this country are low in α-linolenic acid (*e.g.*, significant sources of α-linolenic acid such as soybean oil and rapeseed oil have not been commonly used in the margarine and vegetable oil industry).

Bjerve *et al.* (8) recently showed that plasma phospholipid (PL) concentrations of EPA and DHA can be used to assess the ω3 PUFA status. Within the dietary range of 0.1 to 0.6% of energy of total ω3 PUFA (0.05 to 0.5 g per day of EPA and 0.1 to 0.8 g per day of DHA), there was a rectilinear association between plasma PL EPA and DHA levels and the dietary intake. Previously, plasma fatty acid levels have only been considered as a short-term indicator of dietary intake, whereas long-term intake was thought to be better judged from the fatty acid pattern of tissues or cells such as erythrocytes or white blood cells (9).

The level of ω3 PUFA in tissues such as plasma, white cells and erythrocytes is low relative to the ω6 PUFA in people on typical Western diets (10–13). In addition, the EPA level is substantially lower than that of DHA and, in the case of the plasma PL, it is of the order of 0.2 × the DHA value (10,13). From these data, the following conclusions can be drawn: (i) intake or absorption of EPA is substantially lower than that of DHA, (ii) there is a selective utilization of EPA and/or (iii) there is a selective retention of DHA.

Although it is recognized that dietary change can be associated with rapid changes in the fatty acid composition of plasma lipids (13), the study of plasma lipids can provide useful information about fatty acid dynamics in the body since all dietary lipids pass through the plasma pool(s) before being incorporated in tissue lipids. The purpose of this investigation was to study the accumulation and loss of EPA and DHA in human plasma since it was believed that this might help explain why there was such a difference between the levels of these fatty acids in plasma in subjects on typical Western diets containing low levels of ω3 PUFA.

The design of this experiment was to increase the levels of EPA and DHA in plasma over a two-week period, then

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Abbreviations: CE, cholesteryl esters; DHA, docosahexaenoic acid (22:6ω3); DPA, docosapentaenoic acid (22:5ω3); EPA, eicosapentaenoic acid (20:5ω3); GLC, gas-liquid chromatography; HDL, high density lipoproteins; LCP, long-chain polyunsaturated fatty acids; LDL, low density lipoproteins; PL, phospholipid; PUFA, polyunsaturated fatty acids; TG, triglycerides.

to follow the washout of these PUFA from plasma during the next 4 wk when the subjects were on a diet with very low levels of LCP ω3, thus ensuring that the disappearance from plasma was not confounded by an influx of ω3 LCP from the diet. The fractions chosen for study were the plasma PL, triglycerides (TG) and cholesteryl esters (CE) and the high density lipoprotein (HDL) PL.

MATERIALS AND METHODS

Subjects. Seven healthy, female volunteers (mean age, 30.6 yrs; mean body mass index, 21.4) participated in the study. The protocol for this study was approved by the Deakin University Ethical Review Committee (Geelong, Australia) and informed consent was obtained from the volunteers, all of whom were staff members of the Department of Human Nutrition, Deakin University.

Experimental protocol. The study ran for six weeks. In the first two weeks (ω3 supplementation phase) the subjects consumed their normal diet plus four 1-g capsules daily of MaxEPA (Nature's Way®, Nature's Way Health Products, Chadstone, Australia). Analysis of the oil by capillary gas-liquid chromatography (GLC) using an internal standard of triheptadecanoin (Nu-Chek-Prep, Elysian, MN) established that the concentrations of EPA, DPA and DHA were 137, 21 and 98 mg/g oil, respectively (16.3, 2.5 and 12.1% of total fatty acids); thus the 4 g of fish oil provided 548 mg EPA and 392 mg DHA daily. In the remaining four weeks (ω3 depletion phase), the subjects were advised to reduce the ω3 PUFA content of their diet to a minimum by avoiding fish and other marine products, and vegetable oils and margarines containing α-linolenic acid such as canola oil, and walnuts, red meat and offal meats. They were allowed to consume one egg/week, three meals of lean pork and/or chicken (<100 g/serve) per week and were provided with four vegetarian meals weekly, consisting mainly of lentils and vegetables. The estimated maximum intakes of EPA, DPA and DHA in this latter phase were less than seven mg/d for each ω3 LCP. These figures were calculated from our own data on the PUFA content of Australian foods (14,15). Blood samples were taken before the study began and at weekly intervals between 08:00 and 09:00 following a 10-h overnight fast.

Fatty acid analysis of plasma and HDL lipids. Fasting blood samples were collected in heparinized tubes, stored in ice immediately after collection and centrifuged at 3000 rpm (1700 × *g*, 15 min, 4°C) in a Beckman TJ-6 centrifuge (Beckman Instruments Australia, P/L., Melbourne, Victoria, Australia). Plasma was collected for cholesterol and TG analysis, and aliquots were stored for subsequent lipid extraction. The HDL were separated from a 1.0 mL aliquot of plasma within 2 h of blood collection from other plasma lipoproteins by precipitation with dextran sulfate (Dextralip 50, Sochibo, Velizy Villacoublay, France).

Lipids of whole plasma and the HDL fraction were extracted with chloroform/methanol (2:1, vol/vol) containing 10 mg/L of butylated hydroxytoluene (16). Internal standards of diheptadecanoyl phosphatidylcholine (Sigma, St. Louis, MO), cholesteryl heptadecanoate and triheptadecanoin (Nu-Chek-Prep) were added to the plasma samples and HDL samples prior to lipid extraction. The lipid extracts were separated by thin-layer chromatography (16), and the fatty acid methyl esters of the PL, CE and TG fractions were formed as described previously (16). The fatty acid methyl esters were analyzed by flame-ionization capillary GLC (model GC-9A, Shimadzu, Kyoto, Japan) using a 50 m × 0.32 mm i.d. fused silica (CPSil 88) capillary column (Chrompack, Middelburg, The Netherlands), programmed from 110°C for 3 min to 190°C at 8°C/min with helium as carrier gas at a flow rate of 5 mL/min. Fatty acids were identified by comparison with standard mixtures of fatty acid methyl esters, and the results were calculated using response factors derived from chromatography standards of known composition (GLC-68A and 63B, Nu-Chek-Prep).

Lipoprotein lipid analysis. Concentrations of cholesterol and triglyceride in fasting plasma were measured enzymatically on an autoanalyzer (Hitachi Automatic Analyzer 705, Nissei Sangyo Co. Ltd., Tokyo, Japan) using commercially available kits (Cholesterol CHOD-PAP; Triglyceride GPO-PAP; Boehringer Mannheim, Germany).

Statistical analyses. The results are presented as mean ± SD. The experiment was designed to compare the increase in the ω3 LCP values at week 2, achieved using the MaxEPA supplement, with the initial levels of these PUFA, and to compare the values at the end of the depletion phase (week 6) with the initial values. The statistical significances between means (week 2 *vs.* week 0, and week 6 *vs.* week 0) were analyzed using a paired *t*-test; significant results were taken as $P < 0.05$.

RESULTS

Plasma cholesterol and TG levels. There were no significant changes in the total plasma cholesterol and TG levels during the course of the experiment (Table 1), which is consistent with the results of a 20-wk study using similar low doses of ω3 LCP (17).

Initial fatty acid data. The pre-trial plasma PL fatty acid concentrations of the seven subjects were within the normal range established in this laboratory (Table 2). The mean values for the plasma PL arachidonic acid, EPA and DHA were 9.3, 0.6 and 3.0 (as percent of phospholipid fatty acids), respectively; however, there was a spread of the values for the ω3 LCP, indicating a variation in ω3 intake of the seven subjects prior to the start of this experiment. The EPA values ranged from 0.3 to 1.0 mg/100 mL and the DHA concentrations from 2.0 to 4.5 mg/100 mL. There was no significant correlation between the EPA and DHA values ($r^2 = 0.045$), and the EPA-to-DHA ratio in the plasma PL varied from 0.11 to 0.38 (mean 0.22).

TABLE 1

Mean Concentration of Plasma Cholesterol and Triglyceride During Supplementation and Depletion of Diet with ω3 PUFA

Week	Plasma cholesterol	Plasma *triglyceride*
0	4.50 ± 0.93[b]	0.95 ± 0.25
2	4.37 ± 1.00	0.84 ± 0.49
6	4.40 ± 0.93	1.03 ± 0.47

[a]Results are expressed as mean ± SD deviation. ω3 Polyunsaturated fatty acid (PUFA) supplementation period during weeks 0 to 2; ω3 PUFA depletion period, weeks 3 to 6.
[b]mmol/L.

TABLE 2

Mean Concentration of Fatty Acids in Plasma Phospholipids, Cholesteryl Esters, Triglycerides and High Density Lipoprotein Phospholipids During Supplementation and Depletion of Diet with ω3 PUFA[a]

		Plasma[b]			HDL[b]
	Week	Phospholipid	Cholesteryl ester	Triglycerides	Phospholipid
14:0	0	—	—	1.6 ± 0.8[b]	—
	2	—	—	1.4 ± 0.7	—
	6	—	—	1.4 ± 0.8	—
16:0	0	31.8 ± 6.0	10.0 ± 3.2	16.3 ± 4.5	16.3 ± 2.8
	2	30.8 ± 5.7	10.3 ± 3.0	16.8 ± 10.0	14.0 ± 2.3
	6	31.3 ± 8.6	8.8 ± 1.9	15.6 ± 7.1	15.7 ± 1.7
16:1	0	1.4 ± 0.5	2.8 ± 1.2	3.3 ± 2.0	0.4 ± 0.1
	2	1.1 ± 0.4	2.7 ± 1.1	2.5 ± 1.5	0.4 ± 0.1
	6	1.3 ± 0.6	2.8 ± 1.3	2.8 ± 1.5	0.5 ± 0.2
18:0	0	13.2 ± 2.9	1.3 ± 0.4	3.5 ± 0.8	6.9 ± 1.2
	2	15.0 ± 3.7	1.1 ± 0.4	3.1 ± 1.7	6.1 ± 0.9
	6	13.7 ± 1.9	1.0 ± 0.3	3.0 ± 1.6	6.7 ± 0.9
18:1	0	14.7 ± 2.2	17.1 ± 5.4	26.7 ± 4.1	7.2 ± 1.5
	2	13.1 ± 2.7	17.1 ± 5.8	24.6 ± 12.7	6.1 ± 1.5
	6	14.5 ± 4.0	15.4 ± 4.5	26.6 ± 12.1	6.8 ± 1.2
18:2ω6	0	29.6 ± 5.7	53.2 ± 15.3	10.7 ± 4.0	15.4 ± 3.1
	2	28.0 ± 6.8	52.6 ± 17.4	10.2 ± 6.1	12.6 ± 0.9
	6	32.2 ± 6.8	51.0 ± 12.6	11.4 ± 5.2	14.6 ± 1.5
18:3ω3	0	0.4 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.2 ± 0.1
	2	0.3 ± 0.2	0.5 ± 0.2	0.6 ± 0.5	0.1 ± 0.0
	6	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.1 ± 0.0
20:3ω6	0	3.8 ± 1.1	0.7 ± 0.3	0.2 ± 0.1	2.1 ± 0.2
	2	3.3 ± 1.5	0.6 ± 0.2	0.2 ± 0.1	1.3 ± 0.2
	6	4.3 ± 1.5	0.6 ± 0.2	0.2 ± 0.1	2.0 ± 0.4
20:4ω6	0	10.3 ± 2.5	5.4 ± 2.1	0.6 ± 0.3	5.9 ± 1.0
	2	11.2 ± 2.7	5.6 ± 1.7	0.7 ± 0.4	4.8 ± 0.6
	6	11.8 ± 4.5	4.9 ± 1.9	0.5 ± 0.3	5.3 ± 0.6
20:5ω3	0	0.7 ± 0.3	0.6 ± 0.1	0.1 ± 0.0	0.5 ± 0.3
	2	2.9 ± 0.3	1.9 ± 0.5	0.3 ± 0.2	1.4 ± 0.3
	6	0.8 ± 0.3	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
22:4ω6	0	0.3 ± 0.1	—	0.2 ± 0.1	0.2 ± 0.0
	2	0.3 ± 0.2	—	—	0.1 ± 0.0
	6	0.4 ± 0.2	—	0.1 ± 0.1	0.2 ± 0.0
22:5ω3	0	0.8 ± 0.3	—	0.2 ± 0.1	0.6 ± 0.2
	2	1.4 ± 0.5	—	0.3 ± 0.2	0.7 ± 0.2
	6	1.0 ± 0.3	—	0.2 ± 0.1	0.5 ± 0.2
22:6ω3	0	3.3 ± 1.0	0.5 ± 0.1	0.3 ± 0.2	2.4 ± 0.7
	2	6.3 ± 1.9	0.7 ± 0.2	0.7 ± 0.6	3.2 ± 0.7
	6	4.7 ± 1.3	0.4 ± 0.1	0.3 ± 0.2	2.4 ± 0.5

[a]Results are expressed as mean ± SD. ω3 Polyunsaturated fatty acid (PUFA) supplementation period during weeks 0 to 2; ω3 PUFA depletion period, weeks 3 to 6. Normal values (in 39 females) for 20:5, 1.5 ± 1.0; 22:5, 1.4 ± 0.5; 22:6, 5.8 ± 2.9 (mean ± SD). HDL, high density lipoprotein.
[b]mg/100 mL.

ω3 Supplementation phase. With all fractions studied, there was a consistent and rapid rise in the EPA concentration after supplementation started and, in most fractions, the maximum increase had occurred by week 1. There was a less consistent response in the increase in DHA in the different fractions (Figs. 1-4). The increases in the EPA and DHA concentrations between the start and week 2 were not related to the initial EPA and DHA values in the plasma lipid fractions.

The results for the plasma PL showed that the maximum values for EPA, DPA and DHA were reached at 2 wk after supplementation commenced (Fig. 1); the increases in the concentration of these fatty acids relative to the pre-trial values were 2.2, 0.6 and 3.0 mg/100 mL plasma for EPA, DPA and DHA, respectively. These values represented increases of 4.1, 1.7, 1.9× the initial values, respectively ($P < 0.0001$, 0.019, 0.0024, respectively). There was a stepwise increase in the DHA level from week 1 to week 2 of the supplementation phase compared with the rise in EPA which had plateaued by the end of week 1 of the supplementation.

Similar results were seen for the plasma CE EPA concentration where there was a rapid rise by week 1 (Fig. 2); the increase in EPA in this fraction was by a factor of 3.4 (an increase of 1.3 mg/100 mL) ($P < 0.0003$). The DHA in the CE fraction also rose significantly ($P < 0.019$)

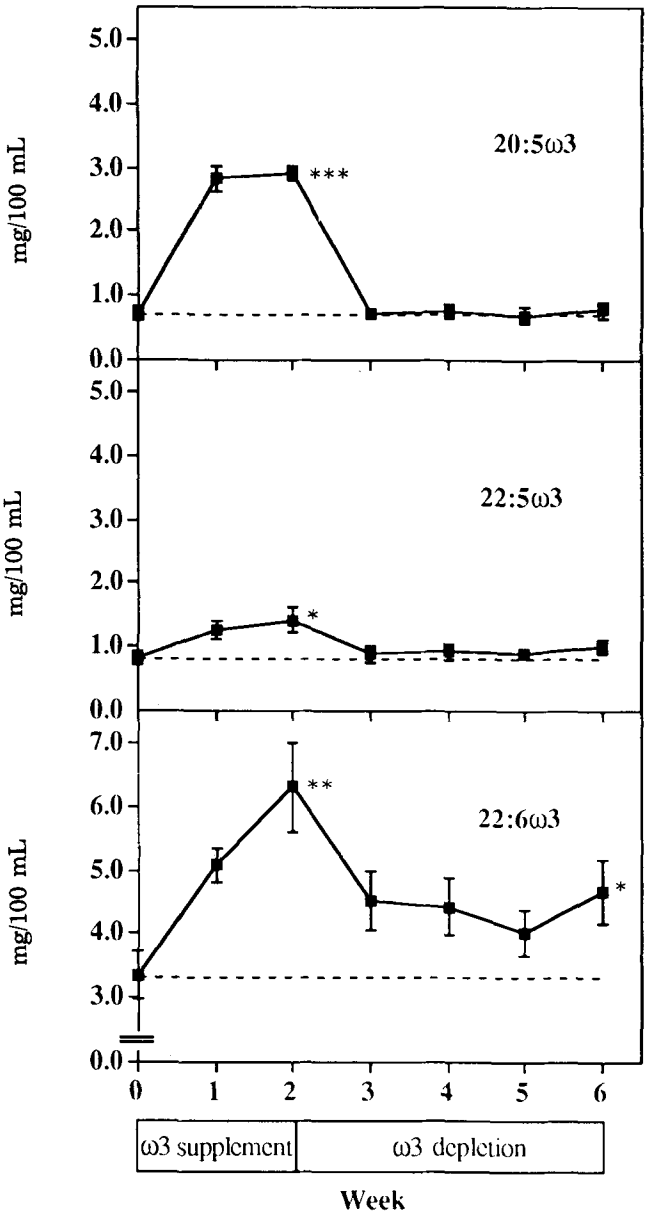

FIG. 1. The concentration (mean ± SD) of ω3 long-chain polyunsaturated fatty acids (PUFA) in plasma phospholipids during supplementation and depletion of diet with ω3 PUFA. Paired *t*-tests were conducted between the week 2 and week 0 samples, and between the week 6 and week 0 samples (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

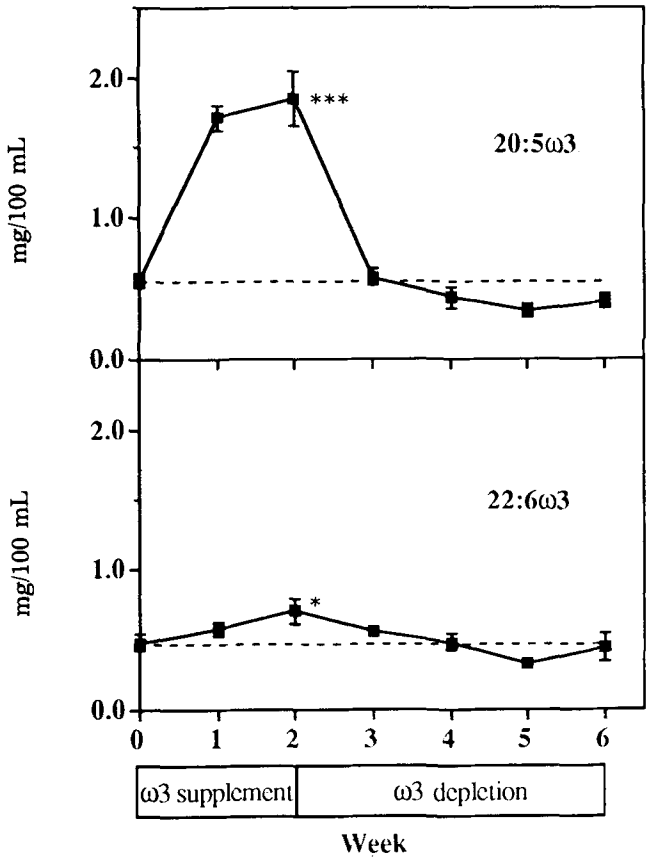

FIG. 2. The concentration (mean ± SD) of ω3 long-chain polyunsaturated fatty acids (PUFA) in plasma cholesteryl esters during supplementation and depletion of diet with ω3 PUFA. Paired *t*-tests were conducted between the week 2 and week 0 samples, and between the week 6 and week 0 samples (*$P < 0.05$, ***$P < 0.001$).

by 0.5 mg/100 mL, a factor of 1.5. There was no detectable DPA in the CE fraction.

In the plasma TG fraction, only the 22 carbon ω3 LCP were evident and only at a low concentration (Table 2); the increases in these fatty acids during the supplementation period were not significant (Fig. 3).

The concentration of ω3 LCP in the HDL PL was about 70% of the total plasma PL ω3 LCP concentration (Table 2). In contrast to the total plasma PL, the rise in all three ω3 LCP in this fraction was maximal after 1 wk of supplementation (Fig. 4). The maximum increases in the concentration of the ω3 LCP relative to the pre-trial values were: EPA 1.3 mg/100 mL ($P < 0.003$), DPA 0.3 mg/100 mL ($P < 0.0083$), DHA 0.8 mg/100 mL ($P < 0.0024$).

ω3 Depletion phase. There was a rapid depletion of EPA and DPA in all of the total plasma and HDL fractions (Figs. 1-4), with the values returning to pre-trial levels within 1 wk.

In the case of DHA, there was a slower depletion than for the other two ω3 LCP, particularly in the total plasma PL (Fig. 1). The DHA concentration had returned to pre-trial values by week 4 for the plasma CE and HDL PL and by week 5 for plasma TG. For the plasma PL there was a substantial drop in the DHA concentration in the first week of depletion (week 3), but no further fall in the remaining 3 wks. For six of the seven subjects examined, the DHA values did not return to the baseline values during the depletion phase. In one subject it reached baseline in weeks 5 and 6. At the end of the experiment, the mean plasma PL DHA concentration was still 40% above the baseline figure ($P < 0.02$) (Fig. 1).

DISCUSSION

The results of this experiment indicate that there were differences in the rate of accumulation and decay of the

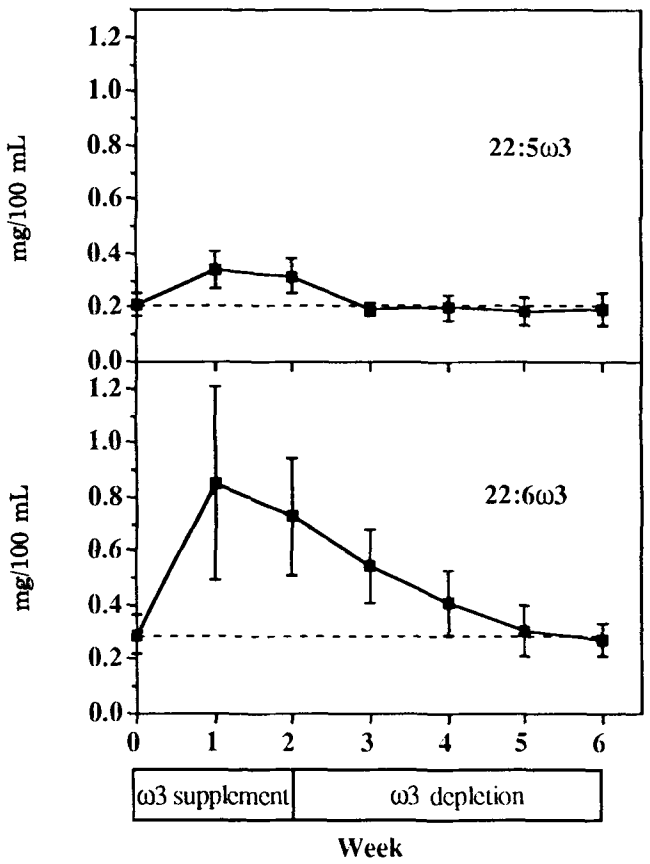

FIG. 3. The concentration (mean ± SD) of ω3 long-chain polyunsaturated fatty acids (PUFA) in plasma triglycerides during supplementation and depletion of diet with ω3 PUFA. Paired *t*-tests were conducted between the week 2 and week 0 samples, and between the week 6 and week 0 samples.

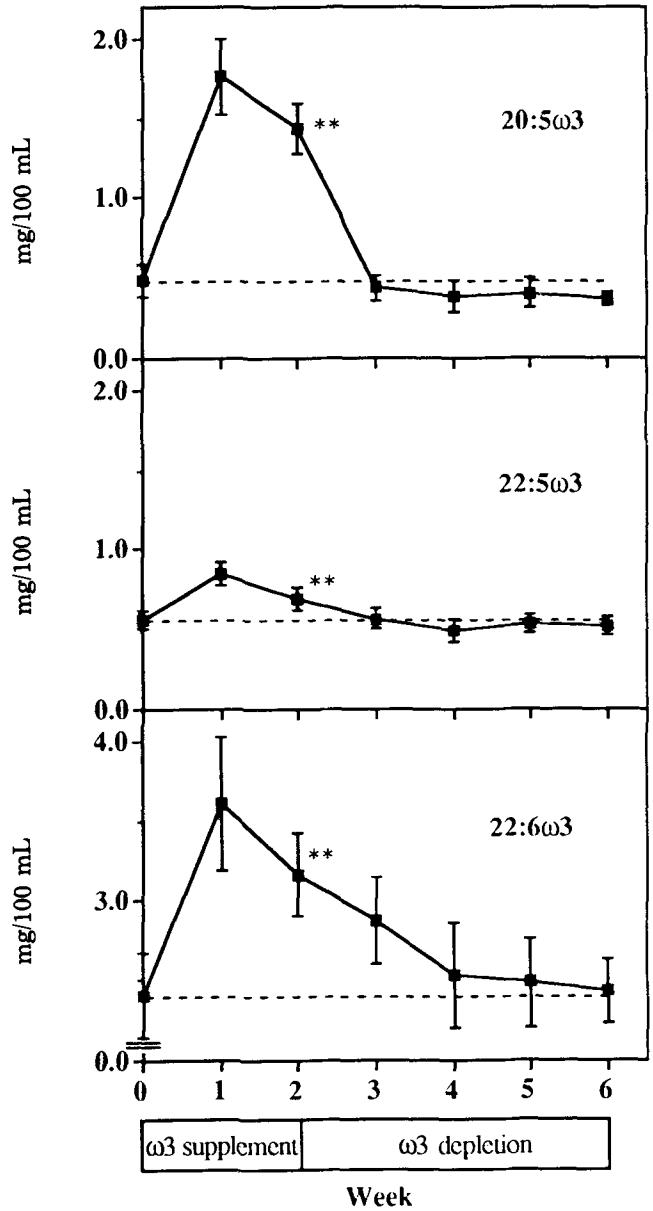

FIG. 4. The concentration (mean ± SD) of ω3 long-chain polyunsaturated fatty acids (PUFA) in high-density lipoprotein plasma phospholipids during supplementation and depletion of diet with ω3 PUFA. Paired *t*-tests were conducted between the week 2 and week 0 samples, and between the week 6 and week 0 samples $^{**}P < 0.01$.

three ω3 LCP in the plasma lipids during the supplementation and depletion phases, respectively. The EPA accumulated in the plasma PL and CE more rapidly than DPA and DHA, and in the depletion phase the EPA and DPA decayed more rapidly compared with DHA, particularly in the plasma PL. The EPA and DPA had returned to pre-trial values within one week in all plasma fractions whereas there was a slower decrease in the DHA concentrations and, in the case of the plasma PL, the DHA still remained above the pre-trial concentration 4 wk after the supplementation had finished.

These results are consistent with those reported by Bronsgeest-Schoute *et al.* (18), where volunteers were given different doses of fish oils (1.4–8.2 g/d of ω3 fatty acids) for 4 wk, and the effects of the plasma lipids were monitored during this period and for a further 2 wk after supplementation ceased. At the completion of the experiment for the three highest dose levels, the EPA, but not the DHA values, had returned to their pre-trail levels in the plasma CE, TG and phosphatidylcholine fractions. Similar results have been reported by others for erythrocytes and platelets (12,19). The slow return of erythrocyte DHA to baseline by comparison with EPA has been noted in particular by Brown *et al.* (20). They reported that 12 wk after fish plus fish oil supplementation had finished in eight volunteers, the degree of retention of erythrocyte EPA was 16% compared with a 44% retention of DHA (values expressed as a percent of the maximum increase on the fish plus fish oil diet). The present study differs from those discussed above, since in none of the previous studies was there a specific reduction in the dietary intake of ω3 LCP during the "washout" phase, thus confounding the possible interpretation of the results for the decay of the ω3 fatty acids.

There was an interesting difference between the disappearance of the ω3 fatty acids from HDL PL and total

plasma PL: in the former case, the DHA values returned to baseline within 2 wk whereas the disappearance from the total plasma was much slower. Although the interrelationships between the PLs in the different plasma lipoproteins are a poorly understood area of study, these results suggest there may be different rates of metabolism for the acyl chains in the PLs of the plasma lipoproteins.

These results also showed differences in the distribution of ω3 LCP in the lipid classes of the plasma and a disproportional accumulation of DHA. Most of the EPA was incorporated into both the PL and CE fractions (44% in PL, 48% in CE), whereas DPA and DHA were mainly found in the PL fraction (86 and 83%, respectively). This partitioning of EPA and DHA into different plasma lipids has been noted previously (18) and might be explained by differences in the positional specificity of EPA and DHA in the chylomicron TG (21,22). There was also a disproportional increase in the DHA in the plasma lipids. For example, at the end of the supplementation phase, the DHA-to-EPA ratio in the plasma lipids was 1.0 (expressed as the increase in DHA in the plasma lipid fractions, PL plus CE plus TG, over the 2-wk supplementation period/increase in EPA) compared with a ratio of 0.7 (DHA-to-EPA) in the supplementary fish oil. The DPA-to-EPA ratio in the plasma lipids was 0.19 compared with 0.15 in the supplement, and the DPA-to-DHA ratio was 0.20 in both the plasma and the supplement. These calculations assume that most of the LCPω3 in the supplementation phase was derived from the fish oil supplement and not from other dietary sources such as fish. A disproportional increase in DHA into various tissues relative to EPA has been reported for rabbits and rats fed fish oils (23,24). In the former article, it was estimated that DHA was three times more likely to be incorporated into adipose tissue than was EPA: the authors speculated that EPA was diverted into alternative metabolic pathways such as oxidation or deposition in other tissues.

The results from this and other studies showing marked differences in the fate of EPA and DHA following ingestion may allow an explanation of the low EPA levels in plasma lipids relative to the DHA levels. These data are consistent with the following possibilities: (i) that EPA is absorbed less effectively than DHA. This is not consistent with the data on the absorption of EPA and DHA from MaxEPA in the rat (22). (ii) That EPA is rapidly removed from the plasma pools to other tissues. This is not consistent with data showing disproportional increases in DHA relative to EPA in a variety of tissues (liver, muscle, adipose) (23,24). (iii) That EPA is β-oxidized faster than DHA. This is consistent with a preliminary report which indicated a more rapid *in vitro* β-oxidation of EPA compared with DHA in rat liver and muscle (25), a significantly greater rate of acylcarnitine synthesis of EPA compared with DHA in rat liver mitochondria (26) and an accelerated loss of α-linolenic acid and EPA compared with DHA in adipose tissue of human subjects undergoing weight loss (27). Gronn *et al.* (28) have indicated that the feeding of fish oil or purified ω3 PUFA to rats stimulates fatty acid oxidation in both mitochondria and peroxisomes and leads to an increased peroxisomal oxidation of ω3 PUFA. This report did not indicate whether there was any difference between the rate of oxidation of EPA and DHA. Zhang *et al.* (29) have reported that in the perfused rat liver, there is significantly less EPA compared with DHA incorporated into very low density lipoprotein lipids which is consistent with a greater of oxidation of EPA in the liver. (iv) That there is a rapid conversion of EPA to DHA. There have been no experiments conducted in humans which have directly addressed this issue. Indirect evidence from experiments of several weeks' duration does not support the view that EPA is rapidly transformed to DPA and DHA in humans. These experiments involved a variety of dietary changes, such as feeding canola oil or lean meat, which induced increases in EPA or EPA and DPA, respectively, in plasma or white cell lipids (13,30). These data do not rule out the possibility of slow conversion or the possibility of any DPA and DHA formed being transferred from the plasma to a tissue such as adipose (27). There must be some conversion of α-linolenic acid to DHA in humans since vegan vegetarians have normal levels of EPA and DHA in their plasma lipids (10), although the direct measurement of metabolism of deuterated α-linolenic acid suggests that the process is very slow (31). (v) That there is a lower intake of EPA than DHA in typical Western diets. Dietary sources of EPA and DHA in typical Western diets such as those consumed in Australia would include fish (EPA-rich for fatty fish and DHA-rich for lean fish, Ref. 32), lean red meat (which contains EPA plus DPA, Ref. 15), offal (which contains EPA, DPA, DHA) and eggs (which contain DHA) (14). Furthermore some EPA and DPA may be produced by the slow conversion of dietary α-linolenic acid to EPA (30,31). It is possible that the dietary intake of EPA is lower than that of DHA in some individuals (*e.g.*, diets containing fish low in fat and therefore DHA-rich, and eggs, which contain DHA but no EPA), but in general the EPA intake on a diet containing a variety of foods should be of the same order as that of DHA, as we have shown previously (13).

In conclusion, the relatively low levels of EPA compared with DHA in human plasma may be the result of a lower intake of EPA and an increased β-oxidation of EPA relative to DHA. One practical outcome of this data, especially for those with low levels of ω3 LCP, is that maintenance of increased EPA levels in plasma (and therefore tissues) would require constant inputs of EPA due to the relatively rapid loss of EPA from tissues.

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Dietary α-Linolenic Acid Alters Tissue Fatty Acid Composition, but Not Blood Lipids, Lipoproteins or Coagulation Status in Humans[1]

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We examined the effect of dietary α-linolenic acid (ALA) on the indices of lipid and coagulation status and on the fatty acid composition of serum and peripheral blood mononuclear cell (PBMNC) lipids in ten healthy men (age 21–37 yr) who consumed all their meals at the Western Human Nutrition Research Center for 126 d. There was a stabilization period of 14 d at the start when all 10 subjects consumed the basal diet (BD) containing 23.4 energy percent (en%) fat and two intervention periods of 56 d each. During the first intervention period, 5 subjects consumed the BD containing 23.4 en% fat, and 5 subjects consumed a diet providing 6.3% calories from α-linolenic acid [flaxseed oil (FSO) diet containing 28.8 en% fat]. Diets were crossed over between the two groups during the second intervention period. Feeding the FSO diet did not significantly alter serum triglycerides, cholesterol, high-density lipoproteins, low-density lipoproteins, apoprotein A-I and apoprotein B when compared to the corresponding values in the subjects fed the BD, nor was there any effect of the FSO diet on the bleeding time, prothrombin time and partial prothrombin time for these subjects. Feeding the ALA-containing diet did cause a significant increase in ALA concentration in serum ($P < 0.001$) and PBMNC lipids ($P < 0.05$). It also caused a significant increase ($P < 0.05$) in the eicosapentaenoic and docosapentaenoic acid contents of PBMNC lipids, and a decrease ($P < 0.01$) in linoleic and eicosatrienoic acid contents of serum lipids. Thus, dietary ALA, fed for 56 d at 6.3% of calories, had no effect on plasma triglyceride or very low density lipoprotein levels or the common risk factors associated with atherosclerosis, although these parameters have been reported by others to be influenced by fatty acids, such as palmitic or linoleic acids, in the diet. Dietary ALA did significantly alter the fatty acid composition of plasma and PBMNC.

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Several studies have examined the effects of oils, usually of marine origin, rich in n-3 fatty acids on the concentration and composition of serum lipids (1,2). Most of these studies have indicated a reduction in serum triglycerides and very low density lipoprotein (VLDL) cholesterol with fish oil feeding. The results of these studies in regard to total serum cholesterol and low-density lipoprotein (LDL) cholesterol have been variable. Some of these inconsistencies may have resulted from differences in the level and duration of fish oil consumption, cholesterol and total fat, as well as the fatty acid composition of the diet. Fish oil consumption also has been reported to increase the bleeding time in human subjects (3,4). In contrast to the many studies conducted with marine oils, only limited studies have been conducted to examine the effects of n-3 fatty acids of plant origin on serum lipids and bleeding time in humans. Flaxseed oil (FSO) is free of cholesterol and contains about 60% α-linolenic acid (ALA, 18:3n-3). Thus, it is the richest source of n-3 fatty acids among plant oils. However, it does not contain any C_{20} or C_{22} n-3 fatty acids usually found in fish oils. Previous studies that have examined the effects of ALA-containing diets on the serum fatty acid composition have shown that humans have a limited capacity to convert ALA to eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (5–7). In the study reported here, we examined the effects of FSO-containing diets on serum lipids and indices of coagulation in ten healthy men. We also examined the effects of FSO-containing diets on the serum and peripheral blood mononuclear cell (PBMNC) fatty acid composition.

MATERIALS AND METHODS

Subjects. Sixteen men were selected to participate in the study. All had a complete physical examination by a physician to rule out any underlying disease. Data regarding only ten of the men who completed the entire study are included in this report. Partial results obtained from the six subjects who dropped from the study were comparable to those obtained from subjects who completed the entire study. Their ethnic backgrounds were as follows: one African-American, one Hispanic, one Filipino, one Iranian and six Caucasians. Nine of these men were soldiers assigned to the Letterman Army Medical Center and the Letterman Army Institute for Research, and the tenth was a civilian working at the Western Human Nutrition Research Center (WHNRC). They ranged in age from 21–37 (mean ± SEM, 27.3 ± 1.9 yr), in weight from 62.7–81.8 (74.6 ± 2.4 kg) and in height from 1.63–1.88 (1.74 ± 0.03 m). The study was approved by the institutional review boards of the U.S. Department of Agriculture and the Letterman Army Medical Center. Informed consent was obtained from all subjects before participation in the study.

Experimental design. The study consisted of an initial 14-d stabilization period and two intervention periods of 56 d each. During the stabilization period, all subjects consumed the basal diet (BD). For intervention period 1 (study days 15–70), five of the subjects continued to

[1]The views expressed in the paper are those of the authors and do not reflect the official policy or position of the Department of Agriculture or Department of Defense, or the U.S. Government.

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; apo A-I, apoprotein A-I; apo B, apoprotein B; BD, basal diet; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; en%, energy %; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; FSO, flaxseed oil; GLC, gas-liquid chromatography; HDL, high density lipoproteins; LA, linoleic acid; LDL, low density lipoproteins; PBMNC, peripheral blood mononuclear cells; VLDL, very low density lipoproteins; WHNRC, Western Human Nutrition Research Center.

consume the BD while the other five were fed an FSO (Omega Nutrition, Inc., Vancouver, British Columbia, Canada) supplemented diet. Subjects in the two groups were matched for age and weight; however, as each subject was fed both diets, the basis used for their distribution into two groups was not critical. The FSO was stored at −20°C and served cold mixed with yogurts, salads, sandwich spreads and vegetables. The diets were crossed over between the two groups for study days 71–126 (intervention period 2). All meals were prepared and consumed at the WHNRC on a 7-d rotating menu for 126 d under the supervision of the dietary staff. The subjects engaged in their normal activities and resided at their own homes. Body weights were maintained constant throughout the study by caloric adjustments and by maintaining the physical activities at the pre-study level.

Diets. Both diets consisted of natural foods except that 100 international units of α-tocopherol was supplemented twice a week to both dietary groups. The mean daily nutrient intake from the BD and FSO diets during the two intervention periods is shown in Table 1. Nutrient intake from the BD was similar for both the stabilization and intervention periods. The BD provided 23.4% energy (en%) as fat, 60.1% as carbohydrates and 16.5% as proteins. The mean daily intake of fat from the basal diet was 77.5 g, of which sunflower oil provided 11.0 g and safflower oil 4.3 g. In the BD, the saturated, monounsaturated and polyunsaturated fatty acids provided 7, 8.6 and 6.3 en%, respectively. The flax diet provided 28.8% energy as fat, 55.0% as carbohydrates and 16.2% as proteins. The mean daily intake of fat in the flax diet was 96.8 g, of which linseed oil provided 31.7 g and safflower oil 4.3 g. Energy from saturated, monounsaturated and polyunsaturated fats in the linseed oil diet was 7.0, 9.6 and 10.6%, respectively. The polyunsaturated/saturated fatty acids ratio of the two diets differed markedly with 0.89 for the basal diet and 1.50 for the linseed oil diet.

The fatty acid composition of the basal and linseed oil diets, in weight percent, are shown in Table 2. The amounts (g) of each fatty acid consumed from the two diets can be calculated from the data presented in Tables 1 and 2. The differences in individual fatty acids consumed each day between the basal and the linseed oil diets were minor, except for ALA. The percentages of energy from ALA, calculated from Tables 1 and 2, are 0.3% for the BD and 6.3% for the flax diet. All other nutrients in both diets were provided at or above the recommended daily allowance.

Laboratory methods. The proximate analysis of the diets was performed by Curtis and Tompkin Ltd. (Berkeley, CA) using standard procedures (8). Fatty acid composition of the diets was determined using capillary gas-liquid chromatography (GLC) (9). Composition of all other nutrients in the diets were calculated from the USDA Handbooks No. 8 and No. 456 (10,11).

Blood from overnight fasting subjects was collected by antecubital venipuncture into vacutainer tubes containing ethylenediaminetetracetate for lipid determinations and no anticoagulant for apolipoprotein determinations. Samples were drawn from subjects in both dietary groups at study days 1, 14, 42, 56, 70, 98, 112 and 126. Samples were always drawn between 7:00 a.m. and 8:00 a.m. and the plasma or serum separated from the cells within 1 h after collection. Specimens that could not be analyzed immediately were stored at 4°C or in accordance with reagent kit manufacturer's instructions. Total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol were analyzed by enzymatic methods (12,13) adapted to the Cobas Fara Centrifugal Analyzer (Roche Diagnostic Systems, Nutley, NJ) (14) using Boehringer-Mannheim reagents (BMC, Indianapolis, IN). HDL cholesterol was

TABLE 1

Mean Daily Nutrient Intakes[a]

Nutrient	Basal diet	Flaxseed oil diet
Energy		
(kcal)	2982 ± 198	3020 ± 131
(J)	12477 ± 828	12236 ± 548
Crude fiber (g)	6.00 ± 0.40	6.66 ± 0.29
Ash (g)	19.00 ± 1.26	17.84 ± 0.77
Protein		
(g)	123.25 ± 8.18	129.53 ± 11.13
(% of energy)	16.54 ± 1.10	16.16 ± 0.70
Carbohydrates		
(g)	435.75 ± 29.73	415.13 ± 17.97
(% of energy)	60.07 ± 3.99	54.98 ± 2.38
Total fats		
(g)	77.50 ± 5.15	96.82 ± 4.19
(% of energy)	23.39 ± 1.55	28.85 ± 1.25
Saturated fatty acids		
(g)	23.23 ± 2.57	23.53 ± 3.23
(% of energy)	7.01 ± 0.78	7.01 ± 0.96
Monounsaturated fatty acids		
(g)	28.57 ± 5.54	32.12 ± 5.93
(% of energy)	8.62 ± 1.90	9.58 ± 1.77
n-6 Polyunsaturated fatty acids		
(g)	19.81	14.8
(% of energy)	6.0	4.3
n-3 Polyunsaturated fatty acids		
(g)	0.81	20.5
(% of energy)	0.3	6.3
Minor fatty acids		
(g)	5.06 ± 0.83	5.91 ± 1.36
(% of energy)	1.53 ± 0.25	1.75 ± 0.41
P/S[b]	0.89	1.50

[a]The proximate analysis was performed on a composite sample prepared for each day of the 7-d menu. $\bar{X}$ ± SEM for ten subjects for each diet (five for each intervention period).

[b]Polyunsaturated/saturated fatty acid ratio.

TABLE 2

Fatty Acid Composition of Experimental Diets (wt%)[a]

Fatty acid	Basal diet	Flaxseed oil diet
12:0	0.65 ± 0.65	0.55 ± 0.50
14:0	1.79 ± 0.57	1.41 ± 0.43
16:0	20.07 ± 1.30	16.05 ± 1.52
16:1n-7	1.35 ± 0.30	1.01 ± 0.26
18:0	7.46 ± 0.80	6.29 ± 0.89
18:1n-9 *trans*	4.69 ± 2.43	3.88 ± 1.83
18:1n-9 *cis*	26.76 ± 2.33	24.29 ± 1.64
18:1n-7	1.77 ± 0.37	1.47 ± 0.30
18:2n-6	25.57 ± 6.66	15.25 ± 3.9
18:3n-3	1.07 ± 0.33	21.18 ± 2.48
24:1	0.70 ± 0.95	1.17 ± 1.60
Unknown and trace	8.12 ± 1.07	7.45 ± 1.40

[a]Data are the mean ± SEM (n = 7).

determined after precipitating LDL and VLDL with phosphotungstate and manganese (15). LDL was calculated using Friedewald's formula (16). Apolipoprotein A-I and B (apo A-I and apo B) were measured by nephelometry using the Beckman Immunochemistry System (Beckman Instruments, Brea, CA) (17). PBMNC were isolated using histopaque-1077 (Sigma Chemical Co., St. Louis, MO) (18), washed three times with phosphate buffered saline and stored at −20°C until direct transmethylation without prior solvent extraction (19). The impurities extracted into the hexane phase after transmethylation were removed by thin-layer chromatography as described by Nelson (20). Fatty acid compositions of the sera were determined by capillary GLC as previously described (21). The column used for serum and PBMNC fatty acid analysis was a 30 m × 0.025 mm fused quartz column coated with SP-2340 (Supelco, Bellefonte, PA). All GLC data were processed with an Perkin-Elmer (Norwalk, CT) model 7500 data station using Perkin-Elmer Chrom 3 software. Bleeding time, prothrombin time and partial prothrombin time were determined at the end of stabilization, and 21 and 56 d after the start of feeding each diet. The bleeding time was determined using a well-established laboratory procedure (22). The prothrombin and partial prothrombin times were measured by a photo optical method using the MLA Electro 700 Coagulation Analyzer (Medical Laboratory Automation, Inc., Pleasantville, NY) (23).

Data analysis. The data were analyzed by analysis of variance using SAS/STAT PROC GLM (24). The cross over design model included effects of order, subject (order), period, diet, time and diet × time using subject (order) as an error term for order. Single degree of freedom, orthogonal polynomial contrast for the time effects were partitioned out of the time and diet × time sources. The significance of the differences between the effects of the two diets were assessed from the P values for the main effect. The diet × time interaction was not significant for any of the measured responses ($P > 0.16$). Changes in the variables examined are considered significant for $P < 0.05$ or otherwise stated. Because the effect of order in which the two diets were fed in the two intervention periods was nonsignificant for all the variables included in this report, the data from the two intervention periods for each diet were pooled. The diet effects were determined analyzing all of the data collected during the study; however, for the ease of presentation, pooled data from all subjects collected at the end of each diet are shown in the Results and Discussion section.

RESULTS AND DISCUSSION

Data regarding the effects of basal and FSO diets on serum concentrations of cholesterol, triglycerides, HDL, LDL, apo A-I and apo B are given in Table 3. None of the response variables were altered by either of the two diets fed to the subjects included in this study. These results differ from those obtained in fish oil studies showing reduction in serum triglycerides and VLDL cholesterol (25–27). This suggests that EPA and DHA are more effective in lowering serum lipids than is ALA. However, the subjects and diets used in our study may also be partly responsible for the lack of effects. The subjects in our study were young and active soldiers whose serum lipids base line values were lower than those of the average public. To exclude the possibility that the low values we measured were in error, we had the cholesterol and triglyceride levels checked in an independent laboratory. The results from the two laboratories were within 5% of each other. The BD used in our study contained very low levels of saturated (7 en%) and total fat (23 en%). Thus, it may not be feasible to lower the serum lipids with the FSO diet or even with fish oils when switching from the type of low-fat diet used in our study. It should also be noted that the FSO diet contained 6 en% extra fat (total 29 en%) above the level of the BD; yet this did not cause an increase in the concentration of any of the serum lipids tested. Although there were minor differences in the concentration of several fatty acids between the two diets, the major difference was in the concentration of ALA (0.3% in BD, and 6.3 en% in FSO diet). It remains possible that the FSO diet would lower serum lipids in hyperlipidemic subjects. The diets and subjects in our study were selected primarily to examine the effects of an FSO diet on the immune response, which was suppressed (18).

TABLE 3

Effects of Basal and Flaxseed Oil Diets on Serum Lipids (mg/dL)[a]

Serum fraction	End stabilization	End flaxseed oil diet	End basal diet
Cholesterol	149.3 ± 8.6	147.0 ± 11.4	153.0 ± 14.1
Triglycerides	79.5 ± 21.3	60.2 ± 14.8	66.1 ± 13.3
HDL	46.1 ± 4.7	48.0 ± 5.8	47.1 ± 4.1
LDL	84.2 ± 12.6	87.2 ± 13.1	90.7 ± 12.4
Apo A-I	118.4 ± 9.1	117.6 ± 9.6	115.9 ± 7.5
Apo B	67.1 ± 11.2	58.9 ± 8.5	68.1 ± 10.6

[a]Data shown are the mean ± SEM (n = 10). During the stabilization period all ten subjects were fed the basal diet. For the next 56 d, five subjects were fed the flaxseed oil diet, and the other five remained on the basal diet. Diets between the two groups were crossed over for the next 56 d. Data were also collected after feeding the two diets for 28 and 42 d and used for analysis of variance. None of the serum lipids were altered by either diet. HDL, high-density lipoproteins; LDL, low-density lipoproteins; apo A-I, apoprotein A-I; apo B, apoprotein B.

The fatty acid compositions of the serum and PBMNC lipids are shown in Tables 4 and 5, respectively. Data presented in Table 4 show that the concentrations of ALA, linolenic acid (LA, 18:2n-6) and eicosatrienoic acid (ETA, 20:3n-3) were significantly ($P < 0.05$) different when the subjects were fed FSO or the BD. With the feeding of the FSO diet, the concentration of ALA in serum lipids increased and that of LA and ETA decreased ($P < 0.05$). The data also show that the levels of other fatty acids including EPA, DHA, and arachidonic acid (AA, 20:4n-6) in serum lipids were not altered by the FSO diet. The data in Table 5 show that the concentration of ALA in PBMNC lipids increased threefold, and that of EPA and docosapentaenoic acid (DPA, 22:5n-3) increased twofold ($P < 0.05$) with the feeding of the FSO diet. In PMBNC lipids, the concentrations of DHA, AA and other fatty acids were not different between the two dietary groups. In serum lipids, an increase in ALA concentration was accompanied by a decrease in the LA and ETA concentrations, while in PBMNC lipids no single fatty acid could be identified whose concentrations decreased when the concentrations of ALA, EPA and DPA increased. This may be because the total concentration of these three fatty acids, even in

TABLE 4

Effects of Basal and Flaxseed Oil Diets on Serum Fatty Acid Composition (wt%)[a]

Fatty acid	Start of study	End flaxseed oil diet	End basal diet
14:0	0.5 ± 0.0	0.6 ± 0.1	0.9 ± 0.1
16:0	24.8 ± 0.9	22.7 ± 0.7	23.5 ± 0.6
16:1	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
18:0	9.7 ± 0.5	10.2 ± 0.4	9.1 ± 0.3
18:1n-9 *trans*	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
18:1n-9 *cis*	15.0 ± 0.2	14.1 ± 0.5	13.8 ± 0.7
18:1n-7	1.6 ± 0.0	1.3 ± 0.1	1.4 ± 0.1
18:2n-6	26.2 ± 1.3	25.4 ± 0.8[b]	28.4 ± 0.6
18:3n-3	0.2 ± 0.0	3.2 ± 0.4[b]	0.5 ± 0.1
20:0	0.5 ± 0.1	0.5 ± 0.0	0.4 ± 0.0
20:3n-6	1.2 ± 0.1	1.1 ± 0.1[b]	1.6 ± 0.1
20:4n-6	6.0 ± 0.6	5.5 ± 0.6	6.2 ± 0.6
20:5n-3	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
22:0	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
22:5n-3	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
22:6n-3	0.8 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
24:0	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
24:1n-9	1.2 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
Unknown and trace	6.6	7.5	6.4

[a]Data shown are the mean ± SEM (n = 9). Fatty acids at a concentration of less than 0.5% are grouped under trace unless they were of specific interest.

[b]Significantly different between basal and flax diets ($P < 0.05$).

the FSO groups, was less than 3%. The fact that the FSO diet contained 21% ALA (Table 2), yet the ALA contents of PBMNC and serum lipids were only 0.6 and 3.2%, respectively, suggests that diet plays only a minor role in determining the ALA concentration of these tissues and that genetic factors have a major regulatory effect.

TABLE 5

Effects of Basal and Flaxseed Oil Diets on Peripheral Blood Mononuclear Cells Fatty Acid Composition (wt%)[a]

Fatty acid	Start of study	End flaxseed oil diet	End basal diet
14:0	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
16:0	20.7 ± 0.7	22.9 ± 2.1	25.3 ± 2.6
16:1	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
18:0	21.6 ± 0.4	21.2 ± 0.9	20.3 ± 0.5
18:1n-9*trans*	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
18:1n-9 *cis*	12.2 ± 0.3	13.2 ± 0.5	12.1 ± 0.3
18:1n-7	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
18:2n-6	5.7 ± 0.3	6.8 ± 0.2	7.6 ± 0.4
18:3n-3	0.2 ± 0.0	0.6 ± 0.1[b]	0.2 ± 0.0
20:0	0.9 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
20:1n-9	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	24.2 ± 0.7	23.8 ± 1.8	23.6 ± 1.3
22:5n-3	0.4 ± 0.1	0.4 ± 0.1[b]	0.2 ± 0.0
22:0	2.8 ± 0.2	2.5 ± 0.2	2.2 ± 0.3
22:5n-3	1.1 ± 0.1	1.6 ± 0.3[b]	0.8 ± 0.1
22:6n-3	1.4 ± 0.1	0.9 ± 0.2	1.1 ± 0.2
Unknown and trace	3.7	2.8	3.1

[a]Data shown are the mean ± SEM (n = 10). For other details see footnote to Table 3. Data were also collected at 28 and 42 d after the start of feeding flaxseed oil and basal diets and used for analysis of variance.

[b]Significantly different between flaxseed oil and basal diet ($P < 0.05$).

The increase in the serum concentration of ALA with the FSO diet is in agreement with previous reports (5,6) showing an increase in plasma ALA concentration when diets were supplemented with this fatty acid. We did not find an increase in EPA and DPA content in serum lipids with the FSO diet, whereas the concentrations of these two fatty acids in PBMNC lipids were increased. This may reflect the fact that triglycerides make up the major portion of the serum lipids, while membrane phospholipids comprise the major part of PBMNC lipids. Previous studies (5–7) on plasma phospholipids, in contrast to our data on total serum lipids, did find an increase in plasma EPA and DHA contents with the feeding of ALA-containing diets. Regardless, whether the total lipids or the phospholipids are analyzed, the conversion of ALA to EPA and DHA appears limited. In our study, we did not find an increase in DHA concentration of PBMNC lipids, even when the EPA and DPA contents were significantly elevated (Table 5). These results suggest that the conversion of ALA to DHA in humans may be restricted at the conversion step from DPA to DHA.

A comparison of the data in Tables 4 and 5 shows minor differences in the concentrations of several fatty acids between serum and PBMNC lipids; however, major differences were observed in the concentrations of AA and LA. The concentration of LA in serum lipids is about 25%, and in the PBMNC lipids it is only about 6%. The AA content of the serum lipids was about 6% and that of the PBMNC lipids was about 24%.

Data regarding bleeding times, prothrombin times and partial prothrombin times are given in Table 6. None of these response variables was altered by either diet. Again these findings are at variance with the results obtained upon feeding of fish oil which caused an increase in bleeding times (3,4). The reasons for the lack of an effect of the FSO diet on these indices of coagulation may be similar to those already discussed for serum lipids.

In conclusion our results show that in the young soldiers studied, the FSO diet did not alter indices of serum lipids or of blood coagulation even when the FSO diet contained 6 en% more fat, which came from ALA. Because this study was done with young subjects on very low-fat diets, it does not rule out the possibility of a serum lipid lowering effect of an FSO diet in hyperlipidemic subjects or in subjects eating high-fat diets. This should be examined in future studies. We did, however, find significant changes in the fatty acid composition of serum and PBMNC lipids in subjects on the FSO diet.

TABLE 6

Effects of Basal and Flaxseed Oil Diets on Bleeding, Prothrombin and Partial Prothrombin Times (min)[a]

Response variable	End stabilization	End flaxseed oil diet	End basal diet
Bleeding time	5.0 ± 0.4	4.5 ± 0.4	4.6 ± 0.4
Prothrombin time	12.2 ± 0.1	12.0 ± 0.2	12.0 ± 0.1
Partial prothrombin time	29.0 ± 0.5	28.9 ± 0.3	28.9 ± 0.6

[a]Data shown are the mean ± SEM (n = 10). For other details see the footnote to Table 3. Data were also collected after 21 d of feeding the two diets and used for analysis of variance. None of the variables in this table were altered by either diet.

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Interrelationship of Stearic Acid Content and Triacylglycerol Composition of Lard, Beef Tallow and Cocoa Butter in Rats

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We investigated modes whereby stearic acid (18:0) exerts a neutral or cholesterol-lowering effect using dietary fats which provided graded levels of 18:0 and distinct triacylglycerol (TAG) profiles. Male Sprague-Dawley rats (150–175 g) were fed diets containing 0.2% cholesterol and 16% fat from corn oil, or from 1% corn oil plus 15% lard (13.2% 18:0), beef tallow (19.2% 18:0) or cocoa butter (34.7% 18:0) for 3 wk, and then killed in a fasted or fed state. Chylomicron (CM) fatty acid profiles suggested reduced absorption of 18:0 with greater 18:0 intake. CM TAG profiles indicated a reduction or loss of two TAG species compared to the TAG profiles of the stearate-rich diets: 1-palmitoyl-2-oleoyl-3-stearoyl glycerol (POS) and 1,3-distearoyl-2-oleoyl glycerol (SOS). Hepatic total cholesterol concentrations were 54–77% lower ($P < 0.01$) in the cocoa butter-fed than the lard- and beef tallow-fed groups. The cocoa butter group showed a significantly lower ratio of high-density lipoprotein esterified/free cholesterol than all other groups. Hepatic stearoyl-CoA and oleoyl-CoA concentrations, the substrate and product for hepatic Δ9 desaturase, were not significantly different for corn oil-fed and cocoa butter-fed groups in spite of a large difference in 18:0 intake. These data suggest that the neutral or cholesterol-lowering effect of 18:0 is not due to hepatic conversion of stearic to oleic acid, and that POS and SOS are poorly absorbed from stearate-rich dietary fats.
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Studies in humans (1–5) and experimental animals (6,7) suggest that ingestion of stearic acid (18:0) has a neutral or hypocholesterolemic effect in contrast to lauric (12:0), myristic (14:0) and palmitic (16:0) acids. Thus, controversy has arisen regarding the classification of stearic acid as a "saturated fat" for dietary recommendations and nutritional labeling purposes (8). This controversy reflects a lack of understanding of the mechanisms responsible for the neutral or hypocholesterolemic effects associated with ingestion of stearic acid. Proposed mechanisms include poor absorption of stearic acid from dietary fat (9–12), rapid conversion of stearic acid to oleic acid by hepatic Δ9 desaturase (13) and effects of the stereospecific triacylglycerol (TAG) structure of stearate-rich dietary fats on their absorption and metabolism (14–16).

Although many studies have examined the effects of feeding stearate-rich fats (commonly defined as having greater than 12% of fatty acids as 18:0), differences in experimental design make it difficult to draw conclusions about the mode(s) of action of stearic acid (17). Using only the fasted state, often a single stearate-rich fat was compared to either a low-stearate saturated fat, an unsaturated fat or both (2,4–7,10–13). Other studies have utilized either synthetic or randomized stearate-rich fats, but these do not reflect the distinct TAG structures of naturally occurring stearate-rich dietary fats (4,9,16). Lastly, some human studies in metabolic ward settings have used liquid formula diets (3,4,17).

The objective of this work was to gain insight into the modes whereby stearic acid exerts its hypocholesterolemic effect. Three naturally occurring dietary fats, lard (13.2% 18:0), beef tallow (19.2% 18:0), and cocoa butter (34.7% 18:0) were fed to provide graded levels of stearic acid, relatively constant amounts of palmitic acid and distinct TAG profiles. Cocoa butter is composed chiefly of three TAG species: 1,3-dipalmitoyl-2-oleoyl glycerol (POP); 1-palmitoyl-2-oleoyl-3-stearoyl glycerol (POS); and 1,3-distearoyl-2-oleoyl glycerol (SOS). It has a relatively simple TAG profile compared to beef tallow and lard which are composed of 7-10 TAG species (15,18,19). We compared both fasting and postprandial plasma and hepatic lipid responses, the fatty acid and TAG profiles of chylomicron (CM) lipids and the levels of hepatic stearoyl-CoA and oleoyl-CoA, the substrate and product for the hepatic Δ9 desaturase system, in rats.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI), 150–175 g, were housed in individual stainless-steel cages with free access to water in a room maintained at 25°C using a 12 h reverse light-dark cycle (lights on 2200–1000 h). The animal facilities and protocols were approved by an institutional animal care and use committee. Rats consumed *ad libitum* one of four diets patterned after the AIN 76A diet (20), containing 16% fat from either 16% corn oil, or 15% lard, 15% beef tallow or 15% cocoa butter with 1% corn oil to prevent essential fatty acid deficiency (Table 1). The fatty acid composition of each diet (Table 2) was determined by capillary gas chromatography with flame-ionization detection (GC-FID). All diets contained 0.2% added cholesterol (Sigma, St. Louis, MO) to provide similar amounts of dietary cholesterol (0.465 mg/kcal). Diets were stored at 4°C and fresh diets were presented to animals biweekly. Food intake was calculated weekly based on a 4-d period and body weight was measured biweekly.

Experimental design. A 4 × 2 (DIET × FED/FASTED) factorial design was used (n = 10). After 3 wk of feeding, all rats were fasted overnight and killed during the early portion of the dark cycle. For each of the four diets, one group of animals was killed in a fasted state (FASTED) while the other group was killed in a postprandial state (FED). The FED rats were fed *ad libitum* for 1 h and killed 1.5 h later. During the 1 h *ad libitum* feeding period, animals consumed 6.0 ± 0.3 g diet. All animals were anesthetized with CO_2 and blood (8–10 mL) was collected by cardiac puncture into syringes containing 1 mg ethylenediaminetetraacetic acid and 0.1 mg gentamicin sulfate

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Abbreviations: ANOVA, analysis of variance; C_n, total acyl carbon number; CM, chylomicron; E/F, esterified/free; EC, esterified cholesterol; FAME, fatty acid methyl esters; GC-FID, gas chromatography-flame-ionization detection; GLM, general linear models; HDL, high-density lipoprotein; LDL, low-density lipoprotein; L, linoleic acid; O, oleic acid; P, palmitic acid; POP, 1,3-dipalmitoyl-2-oleoyl glycerol; POS, 1-palmitoyl-2-oleoyl-3-stearoyl glycerol; SOS, 1,3-distearoyl-2-oleoyl glycerol; TAG, triacylglycerol; TG, triglycerides; VLDL, very low density lipoprotein.

TABLE 1

Composition of Stearate-Rich Diets

Diet composition	
Ingredient[a]	Amount (%)
Casein	20.00
DL-methionine	0.30
Cornstarch	26.89
Sucrose	26.89
Cellulose	5.00
Fat source[b]	15.00
Corn oil[c]	1.00
Mineral mix — AIN 76	3.50
Vitamin mix — AIN 76A	1.00
Choline bitartrate	0.20
Butylated hydroxytoluene (BHT)	0.02
Cholesterol	0.20

[a]All ingredients were obtained from TEKLAD, Harlan Sprague-Dawley, Inc. (Madison, WI) except for cocoa butter, which was obtained from Ambrosia Chocolate (Milwaukee, WI) and cholesterol and BHT, which were obtained from Sigma (St. Louis, MO).
[b]Lard, beef tallow, cocoa butter or corn oil.
[c]To provide essential fatty acids.

TABLE 2

Fatty Acid Profiles of Stearate-Rich Diets

Fatty acid	Corn oil	Lard	Beef tallow	Cocoa butter
	(expressed as % methyl esters)[a]			
14:0	ND	1.3	2.9	ND
16:0	11.4	24.9	24.8	25.4
16:1	ND	2.2	2.8	ND
18:0	2.0	13.2	19.2	34.7
18:1	25.6	44.0	44.1	33.5
18:2	60.1	14.2	6.3	6.3
18:3	1.0	ND	ND	ND
20:4	ND	ND	ND	ND
Ratios				
16:0/18:0	5.7	1.9	1.3	0.7
18:0/18:1	0.1	0.3	0.4	1.0
16:0/18:1	0.4	0.6	0.6	0.8

[a]Values expressed as means for three determinations; ND, not detected.

per mL blood. Plasma was obtained by centrifugation at 4°C for 20 min at 1200 × *g*. Livers were excised, blotted dry, weighed and divided into two portions. One portion was frozen immediately in liquid nitrogen and stored at −70°C for the analysis of individual acyl-CoA esters, and the other portion was stored at −20°C for hepatic lipid analyses.

Lipid analyses. For all FED and corn oil FASTED rats, CM, very low density lipoprotein (d < 1.006 g/mL, VLDL), low-density lipoprotein (d = 1.006–1.050 g/mL, LDL) and high-density lipoprotein (d = 1.050–1.196 g/mL, HDL) were fractionated by sequential ultracentrifugation from 3-mL plasma samples containing 0.56 mM, 5,5′-dithio-*bis*-nitobenzoic acid and 1 mM phenylmethylsufonylfluoride in isopropanol (21). CM were isolated in the top 2 mL after centrifugation for 1.0 h at d < 1.006 g/mL, extracted with hexane/isopropanol (22), evaporated to dryness under nitrogen at 37°C and redissolved in chloroform/methanol (3:2, vol/vol).

Total and free cholesterol were measured in plasma, CM, VLDL, LDL and HDL, and the concentration of esterified cholesterol was determined by difference (21). Triglycerides in plasma (Sigma Triglyceride Kit, No. 336) and lipoprotein samples were also measured. Total protein (VLDL, LDL, HDL), phospholipid and HDL apolipoprotein A-I concentrations were determined as previously described (21). Lipids were extracted from 1-g liver samples with chloroform/methanol (2:1, vol/vol) and assayed for both total and free cholesterol and triglycerides; esterified cholesterol (EC) was determined by difference (21). Individual fatty acyl-CoA esters were analyzed by reverse-phase high-performance liquid chromatography (23).

Triglyceride and cholesteryl ester from the CM lipid extract were isolated on 3 mL aminopropyl (NH_2) Bond Eluts (catalog no. 1212-4038, Varian, Walnut Creek, CA) using a modification of the method of Kalzuny *et al.* (24). An internal standard containing 500 μg each of triheptadecanoin and cholesteryl pentadecanoate (Sigma) was added to a 500 μL aliquot of CM extract (approximately 1–2 mg total lipid). Triglyceride and cholesteryl ester fractions obtained from the Bond Eluts were evaporated to dryness and stored in chloroform/methanol (2:1, vol/vol; 250 μL) until analyzed by GC-FID for either fatty acid or TAG profiles. This procedure was also used to isolate the triglycerides from the diets for TAG profile analyses.

Fatty acid analyses. Both triglyceride and cholesteryl ester fatty acids obtained from CM lipids were transesterified using methanol/benzene (4:1, vol/vol; 2 mL) and acetyl chloride (200 μL) (25). Fatty acid methyl esters (FAME) were stored in benzene at −20°C until analysis by capillary GC-FID. Fatty acids from the diets were transesterified using 10% BF_3/methanol (w/w) reagent (Supelco, Bellefonte, PA) and stored in pentane at −20°C until analyzed. FAME were analyzed on a Varian 3400 gas chromatograph equipped with a flame-ionization detector, a Varian 1093 SPI temperature programmable injector (Palo Alto, CA), a Supelcowax 10 fused silica column, 30 m × 0.32 mm i.d. 0.25 μm film (Supelco) and an In-Board Data Handling System (IBDH™, Sugarland, TX). The injector temperature was progammed from 50 to 250°C at a rate of 100°C/min and held for 15 min during the analysis. The initial column temperature was held for 2 min at 50°C, programmed to 160°C at a rate of 30°C/min, then programmed to 190°C at a rate of 3°C/min and finally programmed to 227°C at a rate of 4°C/min and held for 15 min for a total analysis time of 40 min. The carrier and make-up gases were helium at 2 mL/min and 30 mL/min, respectively. The detector temperature was 300°C. Fatty acids were identified by comparing the retention times with those of known standards (Nu-Chek-Prep, Elysian, MN) and expressed as the weight percent distribution of FAMEs.

TAG profiles. Intact TAG profiles were determined based on total acyl carbon number (C_n) where n is the total number of carbon atoms from fatty acids esterified to the glycerol backbone. Profiles of triglycerides isolated from the diet and composites of CM lipid extract from each FED diet group (n = 8–9) were obtained on a Varian GC-FID equipped with a TAP CB (75% phenyl, 25% methylpolysiloxane) on a WCOT Ultimetal column, 25 M × 0.25 mm i.d., 0.80 mm o.d., 0.10 μm film (Chrompack, Raritan, NY). The injector was isothermal at 360°C, and injection volume was 0.2 μL of a hexane solution (100 μL)

containing triglycerides obtained from Bond Elut separation of lipid classes. The initial column temperature was 290°C, immediately programmed to 330°C at a rate of 10°C/min, finally programmed to 355°C at a rate of 2°C/min and held for 8 min for a total analysis time of 25 min (18,26,27). The carrier gas was helium at 15 psi column pressure; make-up gas was helium at 22 mL/min. The detector temperature was 370°C. Data were acquired using a Waters Baseline 810 Chromatography Workstation connected *via* a Waters System Interface Module (Waters, Milford, MA). Manual baseline correction and reintegration was required for all samples due to the complexity of the chromatograms. Identification of TAG species from the dietary fats and CM TAG was based upon comparison of retention times to a triglyceride standard (catalog no. 178-11, Sigma) and published chromatograms (18,19). Values are expressed as the area percent of each TAG species.

Statistical analysis. Differences among dietary treatment groups were assessed by either one-way or two-way analysis of variance (ANOVA) using the SAS general linear models (GLM) program (SAS, Cary, NC) (28). Group means were considered to be significantly different at $P < 0.05$ as determined by the protected least significant difference technique. Values are expressed as mean ± SEM; means with different superscripts are significantly different.

RESULTS

Growth, food intake and liver weight. Final body weight and growth did not differ across the treatment groups but diet affected mean daily food intake (Table 3). As the stearic acid content of the diet increased, food intake increased significantly and rats fed the cocoa butter diet ate 10% more than rats fed the corn oil diet. Relative liver weight was significantly affected by both DIET and FED/FASTED states such that FED animals had 11% higher liver weights than FASTED animals.

Plasma and hepatic cholesterol and triglycerides. Plasma cholesterol and triglyceride concentrations did not differ among the groups fed different dietary fats but significant differences occurred between the FED and FASTED groups (Table 4). FED groups had 44% greater plasma triglyceride concentrations and 12.5% lower plasma total cholesterol concentrations than FASTED groups. Lower plasma cholesterol concentrations in FED animals were associated with significantly lower esterified, but not free, cholesterol levels. The esterified/free cholesterol (E/F) ratio was significantly lower for the three FED *vs.* FASTED stearate-rich groups while the corn oil group had similar ratios in both FED and FASTED states.

In contrast, dietary fat affected hepatic cholesterol and triglyceride concentrations more strongly than plasma concentrations and a significant DIET × FED/FASTED interaction was observed for hepatic total, esterified and E/F cholesterol concentrations (Table 5). Compared to fasting, hepatic total and EC decreased after consuming a meal containing corn oil or cocoa butter, showed little change after consuming a meal containing lard and increased after consuming a meal containing beef tallow. Among FED groups, cocoa butter showed 54–142% lower ($P < 0.001$) hepatic total and EC levels than lard and beef tallow. Stearate-rich dietary groups had lower hepatic triglycerides than the corn oil group (corn oil = 15.5; lard, beef tallow and cocoa butter = 6.9–11.0 μmol triolein/g liver, $P = 0.0001$, main effects two-way ANOVA).

Hepatic acyl-CoA concentrations. Individual hepatic acyl-CoA concentrations in FED and FASTED animals are summarized in Table 6. Significant DIET × FED/FASTED interaction was observed for stearoyl-CoA (18:0). In FASTED animals stearoyl-CoA (18:0) concentrations generally reflected 18:0 content of the diet (lard < beef tallow < cocoa butter) but were not significantly different. In contrast, in FED animals there was no association between dietary 18:0 and stearoyl-CoA levels. Most notably, FED stearoyl-CoA concentrations did not differ significantly for corn oil (2.0% 18:0) and

TABLE 3

Final Body Weight, Weight Gain, Liver Weight and Mean Food Intake in Rats Fed Stearate-Rich Dietary Fats for Three Weeks[a]

Diet	Final body weight (g)	Weight gain (g/21 d)	Mean food intake (g/d)	Liver weight (g wet tissue/ 100 g body wt)
Fed				
Corn oil	293 ± 6	113 ± 6	13.2 ± 0.3^{c,d}	3.71 ± 0.05^{a,b}
Lard	288 ± 6	109 ± 6	13.5 ± 0.3^{b,c,d}	3.65 ± 0.08^{a,b}
Beef tallow	295 ± 6	115 ± 5	13.9 ± 0.3^{a,c}	3.65 ± 0.04^{a,b}
Cocoa butter	293 ± 6	113 ± 5	14.6 ± 0.3^{a}	3.54 ± 0.05^{a,b,c}
Fasted				
Corn oil	281 ± 6	102 ± 6	13.0 ± 0.3^{d}	3.41 ± 0.03^{b,c,d}
Lard	287 ± 6	107 ± 5	13.2 ± 0.3^{c,d}	3.27 ± 0.07^{c,d}
Beef tallow	285 ± 6	106 ± 5	13.8 ± 0.3^{a,d}	3.23 ± 0.10^{c,d}
Cocoa butter	287 ± 6	107 ± 5	14.3 ± 0.3^{a,c}	3.20 ± 0.05^{d}
Two-way ANOVA (GLM), *P*-values				
Diet	NS	NS	0.0002	0.04
Fed/fasted	NS	NS	NS	0.0001
Diet × fed/fasted	NS	NS	NS	NS

[a]Values are means ± SEM, n = 9–10. Means with different superscripts in the same column are significantly different [$P < 0.05$, two-way analysis of variance (ANOVA), protected least significant difference]; NS, not significant; GLM, general linear models.

TABLE 4

Plasma Cholesterol and Triglyceride Concentrations in Rats Fed Stearate-Rich Dietary Fats for Three Weeks[a]

Diet	Cholesterol				Triglycerides
	Total	Esterified	Free	E/F ratio	
	(mmol/L)				
Fed					
Corn oil	1.58 ± 0.10^{c}	1.27 ± 0.08^{c}	0.31 ± 0.02	4.1 ± 0.1^{b,c,d}	1.28 ± 0.09^{a}
Lard	1.69 ± 0.05^{b,c}	1.33 ± 0.04^{c}	0.36 ± 0.02	3.8 ± 0.1^{d}	1.21 ± 0.09^{a}
Beef tallow	1.78 ± 0.09^{a,b,c}	1.38 ± 0.08^{b,c}	0.40 ± 0.03	3.7 ± 0.3^{d}	1.31 ± 0.07^{a}
Cocoa butter	1.69 ± 0.05^{b,c}	1.32 ± 0.04^{c}	0.37 ± 0.01	3.6 ± 0.1^{d}	1.30 ± 0.06^{a}
Fasted					
Corn oil	1.94 ± 0.14^{a}	1.57 ± 0.10^{a}	0.37 ± 0.05	4.6 ± 0.3^{a,c}	0.92 ± 0.02^{b}
Lard	1.89 ± 0.07^{a,b}	1.57 ± 0.06^{a}	0.33 ± 0.02	4.8 ± 0.2^{a}	0.88 ± 0.04^{b}
Beef tallow	1.86 ± 0.06^{a,b}	1.55 ± 0.05^{a,b}	0.31 ± 0.02	5.1 ± 0.3^{a}	0.86 ± 0.03^{b}
Cocoa butter	2.02 ± 0.12^{a}	1.67 ± 0.12^{a}	0.36 ± 0.01	4.7 ± 0.4^{a,b}	0.91 ± 0.03^{b}
Two-way ANOVA (GLM), *P*-values					
Diet	NS	NS	NS	NS	NS
Fed/fasted	0.0003	0.0001	NS	0.0001	0.0001
Diet × fed/fasted	NS	NS	NS	NS	NS

[a]Values are means ± SEM, n = 9–10. Means with different superscripts in the same column are significantly different [$P < 0.05$, two-way analysis of variance (ANOVA), protected least significant difference]; NS, not significant; E/F ratio, esterified/free; GLM, general linear models.

cocoa butter (34.7% 18:0), but were significantly higher for animals fed lard (13.2% 18:0). Hepatic oleoyl-CoA (18:1) concentrations were affected by DIET but not by FED/FASTED states and, in general, reflected 18:1 dietary intake. The beef tallow dietary group had significantly higher hepatic oleoyl-CoA concentrations than the corn oil group.

Lipoprotein concentration and composition. The concentration and composition of lipoproteins differed significantly among the fed dietary treatment groups. Ingestion of a meal containing cocoa butter resulted in HDL with significantly more (% by wt) protein and free cholesterol and less EC than HDL from animals fed beef tallow or lard (Table 7). The cocoa butter group showed a significantly lower ratio of HDL E/F cholesterol than all other groups. HDL apolipoprotein A-I concentrations did not differ among the groups (data not shown). Taken together, the significantly lower ratio of HDL (Triglyceride + EC)/Protein in the cocoa butter than the beef tallow and lard groups suggests a greater population of lipid-depleted HDL particles with ingestion of cocoa butter than the other stearate-rich fats studied.

The cocoa butter group showed significantly higher concentrations of LDL protein, phospholipid and EC than all other dietary groups which is consistent with a larger number of LDL particles. A different trend was seen in VLDL; beef tallow showed significantly higher VLDL protein, esterified and total cholesterol concentrations and

TABLE 5

Hepatic Cholesterol and Triglyceride Concentrations in Rats Fed Stearate-Rich Dietary Fats for Three Weeks[a]

Diet	Cholesterol				Triglycerides
	Total	Esterified	Free	E/F ratio	
	(μmol/g wet tissue weight)				
Fed					
Corn oil	13.0 ± 0.7^{b,c}	7.9 ± 0.8^{d,e}	3.89 ± 0.20^{d,e}	1.5 ± 0.2^{c,d}	12.7 ± 2.2^{b,c,d}
Lard	16.6 ± 2.0^{a,b}	12.7 ± 1.9^{a,b,c}	3.15 ± 0.43^{d,e}	3.2 ± 0.3^{b,c,d}	9.7 ± 1.7^{b,c,d}
Beef tallow	19.1 ± 1.4^{a,b}	16.0 ± 1.3^{a,b}	4.22 ± 0.20^{b,c,d,e}	4.8 ± 0.7^{a}	8.7 ± 1.0^{b,c,d}
Cocoa butter	10.8 ± 0.9^{c}	6.6 ± 1.0^{d,e}	5.13 ± 0.09^{a,b,c,d}	1.6 ± 0.3^{c,d}	6.0 ± 0.6^{d}
Fasted					
Corn oil	18.0 ± 1.4^{a,b}	12.6 ± 1.3^{b,c}	3.26 ± 0.69^{d,e}	2.3 ± 0.2^{b,c}	18.2 ± 3.2^{a}
Lard	16.1 ± 1.4^{a,b}	12.8 ± 1.1^{a,b,c}	3.75 ± 0.10^{d,e}	2.5 ± 0.8^{b,c}	12.0 ± 1.4^{b,c,d}
Beef tallow	13.8 ± 1.0^{b,c}	10.0 ± 1.0^{b,c,d}	5.72 ± 0.70^{a,b,c}	2.7 ± 0.3^{b,c}	6.2 ± 0.3^{d}
Cocoa butter	13.8 ± 1.0^{b,c}	8.1 ± 1.0^{d,e}	5.40 ± 0.17^{a,b,c,d}	1.6 ± 0.3^{c,d}	7.9 ± 0.9^{c}
Two-way ANOVA (GLM), *P*-values					
Diet	0.004	0.0001	0.0001	0.0001	0.0001
Fed/fasted	NS	NS	NS	NS	NS
Diet × fed/fasted	0.0006	0.0001	NS	0.01	NS

[a]Values are means ± SEM, n = 9–10. Means with different superscripts in the same column are significantly different [$P < 0.05$, two-way analysis of variance (ANOVA), protected least significant difference]; NS, not significant; E/F ratio, esterified/free; GLM, general linear models.

TABLE 6

Acyl-CoA Levels in Hepatic Tissue from Rats Fed Stearate-Rich Dietary Fats for Three Weeks[a]

Treatments	6:0	8:0	10:0	12:0	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:4	Total
						(nmol/g wet tissue wt)							
Fed													
Corn oil	2.4^{a}	1.7	0.5	1.4^{a}	0.3^{d}	0.5^{d}	3.8	1.6^{b}	3.0^{c}	$9.1^{a,b,c}$	15.4^{a}	0.5	38.0
Lard	2.4^{a}	1.7	0.7	$0.8^{a,b}$	1.5^{a}	$1.0^{b,c}$	6.3	3.3^{a}	$5.3^{a,b}$	$12.8^{a,b}$	4.7^{c}	0.7	40.1
Beef tallow	2.4^{a}	1.7	0.6	$1.0^{a,b}$	1.5^{a}	$1.2^{a,b}$	4.7	3.0^{a}	2.5^{c}	$10.1^{a,b,c}$	3.4^{c}	0.8	31.5
Cocoa butter	2.6^{a}	2.0	0.4	0.7^{b}	$1.1^{a,b}$	0.5^{d}	5.7	$2.2^{a,b}$	3.0^{c}	$8.8^{a,b,c}$	3.1^{c}	0.8	28.7
Fasted													
Corn oil	0.9^{b}	1.6	0.3	1.4^{a}	$0.5^{c,d}$	0.3^{d}	3.4	1.5^{b}	$3.6^{b,c}$	5.8^{c}	10.2^{b}	0.9	29.5
Lard	1.2^{b}	1.5	ND	0.6^{b}	$0.9^{b,c}$	$0.8^{c,d}$	5.2	$2.2^{a,b}$	$3.8^{a,b,c}$	$8.3^{b,c}$	4.5^{c}	1.0	29.3
Beef tallow	1.2^{b}	1.5	ND	$1.1^{a,b}$	$1.0^{a,b}$	$1.2^{a,b,c}$	6.3	1.7^{b}	$4.8^{a,b,c}$	13.3^{a}	4.9^{c}	1.0	36.5
Cocoa butter	1.4^{b}	1.9	ND	$0.8^{a,b}$	1.0^{b}	1.6^{a}	5.8	$2.6^{a,b}$	5.9^{a}	$8.7^{a,b,c}$	5.0^{c}	0.9	34.2
Two-way ANOVA (GLM), *P*-values													
Diet	NS	NS	NS	0.004	0.0001	0.0001	NS	0.0185	NS	0.04	0.0001	NS	NS
Fed/fasted	0.0001	NS	NS	NS	0.016	NS	NS	NS	0.04	NS	NS	NS	NS
Diet × fed/fasted	NS	NS	NS	NS	NS	0.0001	NS	NS	0.01	NS	0.004	NS	NS
Pooled SD	0.82	0.49	0.21	0.55	0.46	0.43	2.55	1.07	2.56	4.49	3.06	0.51	9.7

[a]Means with different superscripts under the same column are significantly different, $P < 0.05$, n = 7–9; NS, not significant; ND, not detected; ANOVA, analysis of variance; GLM, general linear models.

a significantly lower proportion of VLDL triglycerides than all other groups (data for LDL and VLDL not shown). CM concentrations generally reflected VLDL, and beef tallow showed similar triglycerides but significantly higher concentrations of total cholesterol than lard and cocoa butter.

Fatty acid profiles of CM triglyceride and cholesteryl esters. The fatty acid composition of CM triglyceride is summarized in Table 8. Cocoa butter ingestion resulted in significantly greater CM triglyceride 18:0 (% methyl ester), and beef tallow ingestion resulted in significantly greater 18:1 than all other diets. The corn oil dietary group had significantly less 16:0, 18:0 and 18:1 but significantly more 18:2 and 20:4 than the stearate-rich dietary groups. The fatty acid composition of CM cholesteryl ester followed similar trends but the proportion of 18:0 did not differ among the dietary groups (data not shown). CM cholesteryl ester also contained a large (35.6–47.1%) proportion of arachidonic acid for all dietary groups.

Among the stearate-rich dietary groups, lower 18:0/18:1 ratios were noted in CM TAG compared to the corresponding diets: lard, 0.15 *vs.* 0.30; beef tallow, 0.16 *vs.* 0.40; and cocoa butter, 0.35 *vs.* 1.0, respectively. Regression analysis showed a strong linear relationship ($r^2 = 0.985$, n = 4, $P < 0.01$) between the 18:0 content of CM triglycerides and 18:0 content of the diets but an insignificant correlation ($r^2 = 0.723$, n = 4, $P > 0.1$) between the 18:1 content of CM triglyceride relative to dietary 18:1 (Fig. 1). The proportion of CM 18:1 was significantly greater in the beef tallow than the lard dietary group, although lard and beef tallow diets did not differ in the proportion of dietary 18:1.

TAG profiles of diets and CM based on C_n. Each diet displayed a distinct TAG profile according to separation by C_n (Tables 9–12 and Figs. 2 and 3). Cocoa butter had the simplest profile, with only three major TAG species at C_{50}, C_{52} and C_{54}. Lard and beef tallow TAG contained primarily C_{50}, C_{52} and C_{54}, but the degree of unsaturation was greatly increased compared to cocoa butter as indicated by longer retention times on the polar column. Beef tallow also had more C_{48} species than the other stearate-rich diets.

TABLE 7

Plasma HDL Composition (d = 1.050–1.196 g/mL) in Rats Fed Stearate-Rich Dietary Fats[a]

	Corn oil-fasted	Corn oil-fed	Lard	Beef tallow	Cocoa butter	Pooled SD
			(% by wt)			
Protein	36.2 ± 0.7	35.0 ± 0.6^{a}	32.9 ± 0.6^{b}	31.5 ± 0.6^{b}	35.4 ± 0.6^{a}	1.9
Phospholipid	16.9 ± 0.8	18.4 ± 0.6^{b}	$20.3 \pm 0.7^{a,b}$	21.4 ± 0.6^{a}	$20.2 \pm 0.7^{a,b}$	2.0
Cholesterol						
Esterified	22.5 ± 0.8	22.3 ± 0.7^{a}	23.6 ± 0.7^{a}	23.0 ± 0.7^{a}	19.9 ± 0.7^{b}	1.9
Free	3.9 ± 0.2	3.1 ± 0.2^{b}	2.6 ± 0.2^{c}	2.4 ± 0.2^{c}	4.6 ± 0.2^{a}	0.5
Total	26.4 ± 0.7	25.4 ± 0.8	26.2 ± 0.6	25.4 ± 0.5	24.5 ± 0.7	1.8
Triglycerides	20.5 ± 1.1	21.2 ± 0.9	20.7 ± 1.0	21.7 ± 0.9	19.9 ± 1.0	2.9
			(ratio)			
E/F cholesterol	3.8 ± 0.6	4.3 ± 0.2^{b}	5.5 ± 0.3^{a}	5.9 ± 0.4^{a}	2.6 ± 0.2^{c}	0.8
(TG + EC)/Protein	0.94 ± 0.03	$1.00 \pm 0.08^{a,b}$	1.06 ± 0.03^{a}	1.12 ± 0.03^{a}	0.90 ± 0.02^{b}	0.14

[a]Values are means ± SEM, n = 6–9. Means with different superscripts in the same row are significantly different ($P < 0.05$, protected least significant difference). E/F, esterified to free ratio; EC, esterified cholesterol; TG, triglycerides; HDL, high-density lipoprotein.

TABLE 8

Fatty Acid Composition of Chylomicron Triglyceride in Rats Fed Stearate-Rich Dietary Fats for Three Weeks[a]

Fatty acid	Corn oil	Lard	Beef tallow	Cocoa butter	Pooled SD
			(expressed as % methyl esters)		
14:0	0.2 ± 0.1[b]	0.4 ± 0.2[b]	1.8 ± 0.1[a]	0.4 ± 0.1[b]	0.3
14:1	0 ± 0[b]	0 ± 0[b]	0.1 ± 0.0[a]	0 ± 0[b]	0.1
16:0	13.5 ± 0.3[c]	24.5 ± 0.9[a]	24.2 ± 0.5[a,b]	22.9 ± 0.3[b]	2.5
16:1	0.2 ± 0.1[c]	1.1 ± 0.3[b]	2.2 ± 0.1[a]	0.4 ± 0.1[c]	0.4
18:0	2.1 ± 0.2[c]	7.1 ± 0.3[b]	8.3 ± 0.3[b]	16.4 ± 1.0[a]	1.6
18:1	24.2 ± 0.5[c]	47.3 ± 1.1[b]	51.9 ± 0.8[a]	47.7 ± 0.8[b]	2.4
18:2	53.8 ± 0.8[a]	16.3 ± 0.4[b]	9.0 ± 0.4[c]	9.5 ± 0.2[c]	1.4
18:3	0.6 ± 0.1	0.6 ± 0.4	0.1 ± 0.1	0.2 ± 0.0	0.6
20:0	0.1 ± 0.0[b]	0 ± 0[b]	0.1 ± 0.1[b]	0.4 ± 0.1[a]	0.2
20:1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2
20:2	0.2 ± 0.1[a]	0.2 ± 0.1[a]	0.0 ± 0.0[b]	0 ± 0[b]	0.1
20:3	0.2 ± 0.1[a]	0 ± 0[b]	0.0 ± 0.0[b]	0 ± 0[b]	0.0
20:4	4.6 ± 0.7[a]	2.3 ± 0.4[b]	2.0 ± 0.3[b]	1.9 ± 0.3[b]	1.3
Ratios					
16:0/18:0	6.5 ± 0.4[a]	3.5 ± 0.2[b]	2.9 ± 1.0[b]	1.4 ± 0.1[c]	0.595
18:0/18:1	0.09 ± 0.01[c]	0.15 ± 0.01[b]	0.16 ± 0.01[b]	0.35 ± 0.03[a]	0.042

[a]Values expressed as means ± SEM, n = 8-9. Means with different superscripts in the same row are significantly different ($P < 0.05$, protected least significant difference).

Only the stearate-rich fats showed changes in the profiles of CM compared to the TAG profiles of the diets (Tables 9-12 and Figs. 2 and 3). Among stearate-rich fats, CM TAG were relatively enriched in unsaturated species within a TAG C_n (Tables 10-12 and Figs. 2 and 3), and the enrichment became more pronounced with increased dietary stearate content. Cocoa butter CM TAG changed most dramatically, with the complete disappearance of two major dietary TAG species, POS and SOS, and formation of several new, more unsaturated species (Fig. 3). Similarly, POS decreased (but was still present) in both lard and beef tallow CM TAG while SOS decreased in beef tallow CM TAG (Fig. 2). Both lard and beef tallow CM TAG were enriched in more unsaturated species at C_{52} and C_{54} but, unlike cocoa butter, these TAG species were also present in the dietary fat.

DISCUSSION

This study provides new information about the effects associated with ingestion of stearic acid during both fasting and postprandial states in the rat. We compared consumption of three naturally occurring, stearate-rich dietary fats providing graded levels of 18:0 and distinct TAG profiles. Our data provide new evidence that both increased 18:0 content and TAG composition influence absorption and intestinal processing of 18:0. For example, a TAG species, identified as POS, was poorly absorbed from all stearate-rich diets while another TAG species, identified as SOS, was poorly absorbed from both the beef tallow and cocoa butter diets. Mattson *et al.* (9) observed that in rats fed a series of TAG isomers, 18:0 esterified at either the *sn*-1 or *sn*-3 position was released as the free acid and poorly absorbed in the presence of calcium and magnesium. Our CM TAG profile data are consistent with

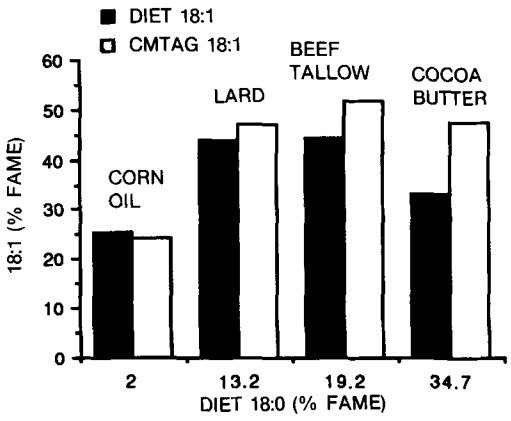

FIG. 1. Relationship between dietary and chylomicron triacylglycerol (CM TAG) oleic acid (18:1) as a function of dietary stearic acid (18:0). Differences between dietary 18:1 and CM TAG 18:1 increased in proportion to the 18:0 content of the diets. Values are weight percent of fatty acid methyl esters (FAME).

TABLE 9

Comparison of Triacylglycerol (TAG) Composition of Diet and Chylomicron from Rats Fed a Corn Oil Diet

Peak[a]	TAG[b]	Diet (area %)	Chylomicron (area %)
1	C48	ND	1.2
2 (POP)[c]	C50	1.4	3.4
3 (PLP)[c]		3.4	4.8
4 (POO)[c]	C52	5.9	5.9
5 (PLO)[c]		14.7	20.1
6 (PLL)[c]		18.2	20.5
7 (OOO)[c]	C54	4.2	1.4
8		1.8	1.4
9 (OLO)[c]		13.9	11.7
10 (OLL)[c]		19.9	17.8
11 (LLL)[c]		14.7	8.6

[a]Refers to order of elution of major chromatographic peaks.
[b]TAG total acyl carbon number.
[c]Identification based on reference to literature (18,19); ND, not detected; P, palmitic acid; O, oleic acid; L, linoleic acid.

TABLE 10

Comparison of Triacylglycerol (TAG) Composition of Diet and Chylomicron from Rats Fed a Lard Diet

Peak[a]	TAG[b]	Diet (area %)	Chylomicron (area %)
1	C48	0.4	1.4
2		0.8	1.4
3 (PPS)[c]	C50	1.6	1.9
4 (POP)[c]		7.6	11.4
5 (PLP)[c]		4.8	7.4
6		0.6	1.4
7	C52	1.4	0.8
8 (POS)[c]		22.2	7.3
9 (POO)[c]		31.1	30.2
10 (PLO)[c]		10.4	16.5
11		2.1	2.5
12	C54	1.3	0.8
13 (SOO)[c]		4.1	3.1
14 (OOO + SLS)[c]		4.1	4.6
15 (SLO)[c]		2.3	2.4
16 (OLO)[c]		2.5	3.8
17		1.1	0.8

[a]Refers to order of elution of major chromatographic peaks.
[b]TAG total acyl carbon number.
[c]Identification based on reference to literature (18,19); P, palmitic acid; L, linoleic acid; O, oleic acid; S, stearic acid.

TABLE 12

Comparison of Triacylglycerol (TAG) Composition of Diet and Chylomicron from Rats Fed a Cocoa Butter Diet

Peak[a]	TAG[b]	Diet (area %)	Chylomicron (area %)
1	50	0.4	3.1
2 (POP)[c]		16.6	9.6
3		1.4	3.2
4	52	ND	2.6
5		ND	13.9
6 (POS)[c]		41.1	ND
7		2.6	28.2
8		2.6	ND
9		0.5	8.9
10	54	ND	5.6
11 (SOS)[c]		27.5	ND
12		3.0	10.1
13		1.5	6.0
14		ND	3.2
15		ND	2.6

[a]See Figure 3 for peak identification.
[b]TAG total acyl carbon number.
[c]Identification based on reference to literature (18,19); ND, not detected; P, palmitic acid; O, oleic acid; S, stearic acid.

this latter observation because 18:0 is esterified primarily at either *sn*-1 or *sn*-3 position in lard, beef tallow and cocoa butter (29), and we observed lower absorption of both SOS and POS species.

Additional evidence for reduced 18:0 absorption is suggested by the observation that rats fed stearate-rich diets showed increased food intake but not increased growth (Table 3). The lower CM than diet TAG 18:0/18:1 ratios, especially in the cocoa butter dietary group, also suggest reduced absorption of 18:0 relative to 18:1 with greater dietary 18:0 intake (10,12,21). These data are supported by the results of another experiment using diets identical to those in the present study where we noted that apparent lipid digestibility decreased with increased dietary 18:0 (30). Apparent lipid digestibility was: cocoa butter, 78% < beef tallow, 82% < lard, 90% < corn oil, 94% ($P < 0.001$).

Other explanations for the lower 18:0/18:1 ratios observed in CM TAG relative to corresponding diets include conversion of dietary 18:0 to 18:1 by intestinal Δ9 desaturase and the contribution of endogenous fatty acids by the intestine prior to reassembly of CM TAG (31,32). Giron *et al.* (31) observed higher Δ9 desaturase activity in duodenal mucosa than in the liver. Perhaps the small intestine plays a regulatory role in fatty acid metabolism by modifying the degree of saturation of dietary fatty acids and TAG structure prior to CM transport out of the enterocyte.

The greater sensitivity of hepatic than plasma cholesterol concentrations to dietary fat was due to differences in fed groups. Differences in the FED response (*i.e.*, increased or decreased hepatic cholesterol concentrations after a meal) to dietary stearate-rich fats emphasize the important role of the intestine in cholesterol metabolism. Among fed rats, hepatic total cholesterol concentrations were 54–77% lower in the cocoa butter than the lard and beef tallow dietary groups. These data support a liver cholesterol-lowering effect of cocoa butter compared to beef tallow and lard.

In general, our research confirms previous observations of differences in hepatic but not plasma total cholesterol concentrations in rats fed stearate-rich *vs.* unsaturated dietary fats and sampled after an overnight fast (6,7,21). A longer feeding period and/or larger amounts of dietary fat, cholesterol and cholic acid may be needed to detect differences in plasma cholesterol given the rats' ability to regulate plasma cholesterol concentrations. For example, Morrissey *et al.* (33) noted that 60 d of feeding 10, 20 or 30% cocoa butter diets to male Sprague-Dawley rats

TABLE 11

Comparison of Triacylglycerol (TAG) Composition of Diet and Chylomicron from Rats Fed a Beef Tallow Diet

Peak[a]	TAG[b]	Diet (area %)	Chylomicron (area %)
1	C48	2.7	1.4
2		4.4	1.8
3 (PPS)[c]	C50	3.7	2.3
4 (POP)[c]		10.6	10.2
5		5.4	8.6
6 (PSS)[c]	C52	2.9	1.0
7 (POS)[c]		13.8	6.2
8 (POO)[c]		23.5	31.2
9		3.6	11.0
10 (SOS)[c]	C54	4.6	1.5
11 (SOO)[c]		8.6	4.7
12 (OOO)[c]		4.3	9.6
13		0.6	2.6

[a]See Figure 2 for peak identification.
[b]TAG total acyl carbon number.
[c]Identification based on reference to literature (18,19); P, palmitic acid; S, stearic acid; O, oleic acid.

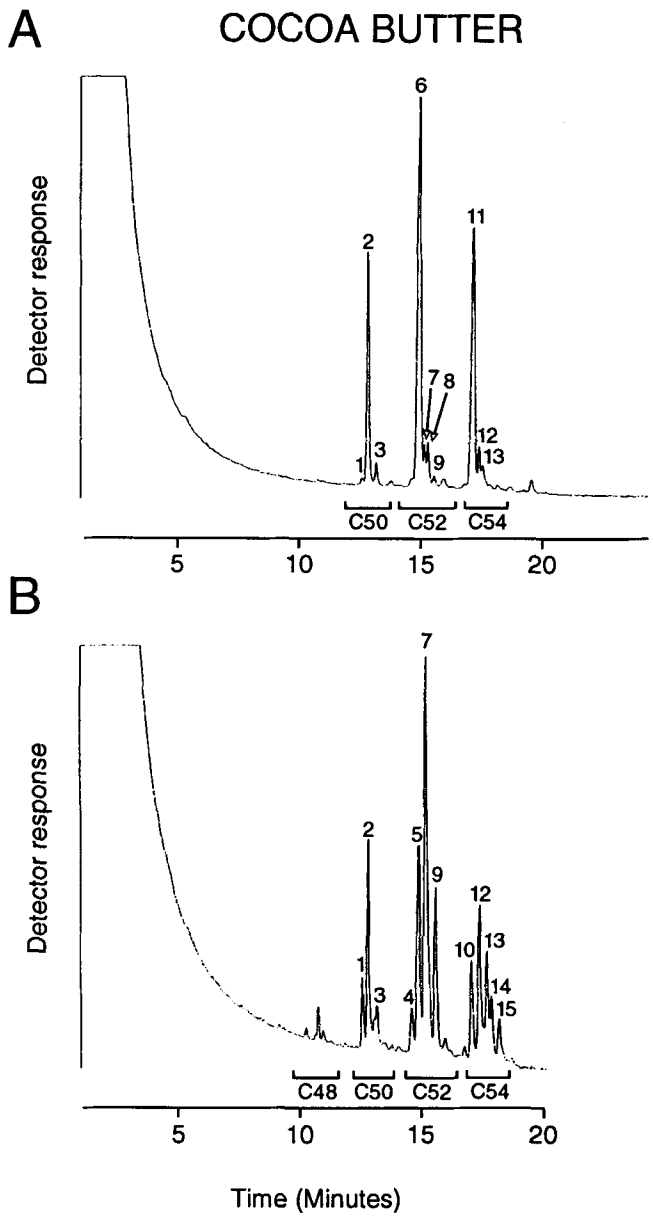

FIG. 2. A: Gas-liquid chromatogram of intact triacylglycerol (TAG) species according to total acyl carbon number (C_{46}-C_{54}) from a 15% beef tallow + 1% corn oil diet. Column: TAP CB on WCOT Ultimetal (25 m × 0.25 mm, i.d.). Temperature: 290°C, immediately programmed to 330°C at 10°C/min, then programmed to 355°C at 2°C/min and held for 8 min. Carrier gas: helium (15 psi). Peak identification: 1,2. unknown; 3. PSS; 4. POS; 5. unknown; 6. PSS; 7. POS; 8. POO; 9. unknown; 10. SOS; 11. SOO; 12. OOO; and 13. unknown; where P, palmitic acid; O, oleic acid; L, linoleic acid; and S, stearic acid. B: Gas-liquid chromatogram of intact TAG species from chylomicrons in rats fed a 15% beef tallow + 1% corn oil diet. Refer to above for peak identification (Refs. 18,19). See Table 11 for area percent.

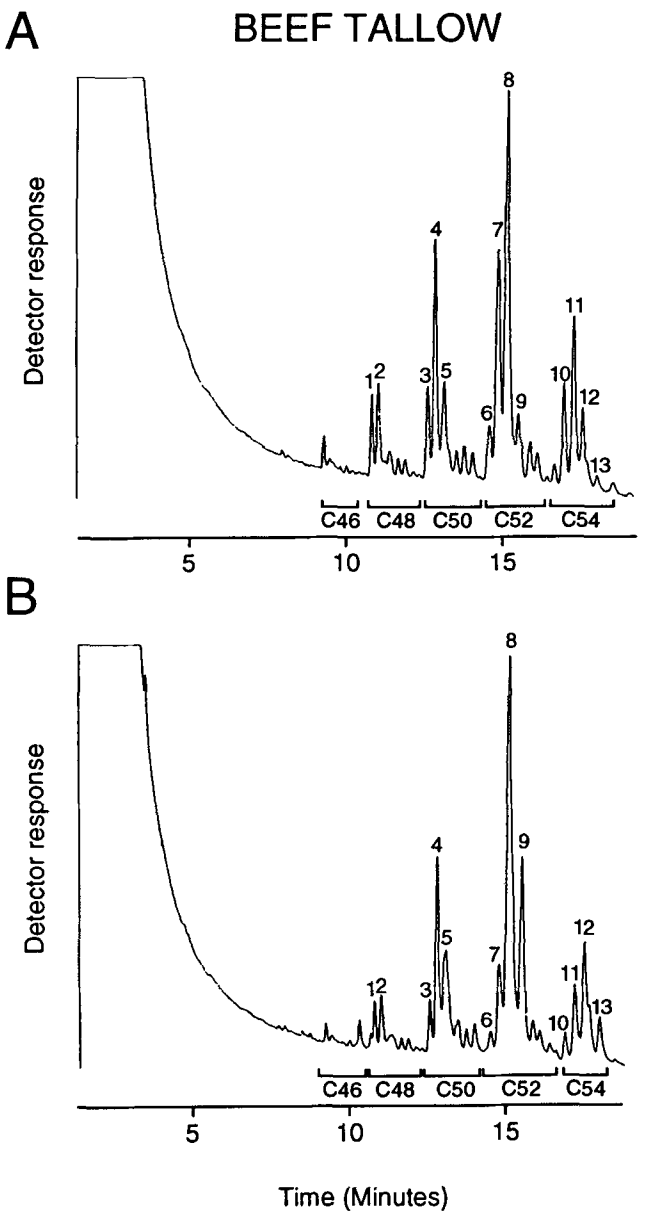

FIG. 3. A: Gas-liquid chromatogram of intact triacylglycerol (TAG) species from a 15% cocoa butter + 1% corn oil diet. Peak identification: 1. unknown; 2. POP; 3. unknown; 6. POS; 7-9. unknown; 11. SOS; and 12,13. unknown. B: Gas-liquid chromatogram of intact TAG species from chylomicrons in rats fed a 15% cocoa butter + 1% corn oil diet. Peak identification (Refs. 18,19): 1. unknown; 2. POP; 3-5,7,9,10,12-15. unknown. See Table 12 for area percent.

was needed to detect a significant decrease in plasma cholesterol concentrations compared to corn oil.

Hepatic concentrations of stearoyl-CoA and oleoyl-CoA provide indirect evidence that little hepatic conversion of 18:0 to 18:1 occurred during feeding of stearate-rich diets (Table 6). Stearoyl-CoA and oleoyl-CoA concentrations were not significantly different for corn oil (2.0% 18:0) and cocoa butter (34.4% 18:0) dietary groups during feeding. If hepatic Δ9 desaturase was rapidly converting dietary 18:0 to 18:1, a higher concentration of hepatic stearoyl-CoA and oleoyl-CoA would be predicted for the cocoa butter than for the corn oil dietary group. Hepatic Δ9 desaturase has been well studied in rats and mice (34-36). Ntambi (36) noted that diets supplemented with essential fatty acids repressed induction of Δ9 desaturase mRNA in mouse liver following fasting and refeeding.

Since our diets contained 1% corn oil, this may explain the apparent lack of hepatic Δ9 desaturase activity suggested by hepatic oleoyl-CoA concentrations.

In conclusion, this study demonstrates that both stearic acid content and TAG composition of stearate-rich dietary fats influence intestinal processing and postprandial hepatic and lipoprotein cholesterol concentrations in the rat. Our data are consistent with reduced absorption of stearate from naturally ocurring stearate-rich dietary fats. Hepatic stearoyl-CoA and oleoyl-CoA concentrations do not suggest rapid conversion of dietary stearate to oleate during feeding. Further research on intestinal Δ9 desaturase and the possible regulatory role of the intestine may provide useful insight into the effects associated with ingestion of stearate-rich dietary fats. Given the distinct TAG profiles of naturally occurring stearate-rich dietary fats, it may not be prudent to make generalized statements about these fats based on stearic acid content alone.

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Cholesterol Synthesis and Esterification in Isolated Enterocytes: Regulation by Cholesterol and Cholestyramine Feeding

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The purpose of the present study was to investigate the physiological control of the main regulatory enzymes of cholesterol metabolism in isolated enterocytes obtained from chick duodenum, jejunum and ileum. Cholesterol feeding resulted in an inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and mevalonate 5-pyrophosphate decarboxylase, while cholestyramine feeding increased reductase activity in all the regions studied and decarboxylase activity only in duodenum. Cholesterol feeding markedly increased acyl-CoA:cholesterol acyltransferase, but the effects of cholestyramine were less clear. The effects on transferase activity cannot be due to differences in the availability of acyl-CoA as exogenous substrate as no significant differences were found in acyl-CoA hydrolase activity after any of the dietary treatments. The effects of cholesterol feeding were related to changes in the cholesterol content of epithelial cells, whereas in the case of cholestyramine this relationship was less apparent. *Lipids 28,* 549–553 (1993).

The rate of *de novo* cholesterol synthesis in the intestine is second only to that in the liver, and, in some animal species, it may contribute more cholesterol to the body than does the liver (1). However, the regulatory role of dietary cholesterol on cholesterol biosynthesis in the intestine has been questioned. One of the early studies that did not show a regulatory effect of dietary cholesterol on cholesterol synthesis in the intestine was that of Dietschy and Siperstein (2), and these observations were confirmed in a more recent study (3). Likewise, Shefer *et al.* (4) were unable to suppress the activity of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in intestine of rats fed a diet supplemented with 2% cholesterol. There are species-related differences in the response of intestinal cholesterol biosynthesis to dietary cholesterol. For example, in the hamster (5), guinea pig (6), rabbit (7) and dog (8), diets supplemented with cholesterol were found to readily down-regulate cholesterol biosynthesis in the intestine. We also have reported an inhibitory effect of cholesterol feeding on chick intestine HMG-CoA reductase (9).

Many technical difficulties exist in the use of the intestine as an experimental model to study the regulation of cholesterol metabolism. Data from one report are difficult to compare with data from another. In addition to the known species differences, distinct functional differences along the length of the small bowel also exist (1).

Bile acids regulate intestinal cholesterol metabolism. The binding of bile acids by resins in the lumen of the intestine (10) or surgical diversion of bile from the intestine (10,11) can cause a marked stimulation of cholesterol synthesis and a decrease in cholesterol esterification in the intestine (12). Rat HMG-CoA reductase of both liver and intestine appears to be stimulated after cholestyramine feeding (13,14). Similarly, cholestyramine feeding increased chick liver and intestine reductase activity throughout postnatal development (9).

Although HMG-CoA reductase is considered the rate-limiting enzyme for cholesterogenesis in liver and other tissues (15,16), the existence of a secondary site of regulation at the step catalyzed by mevalonate 5-pyrophosphate (MVAPP) decarboxylase has been suggested (17–20). We have shown that cholesterol or cholestyramine feeding produced similar effects on MVAPP decarboxylase from chick intestine as those observed on HMG-CoA reductase, while other mevalonate-activating enzymes were not affected (21,22). Likewise, there is now substantial evidence that acyl-CoA: cholesterol acyltransferase (ACAT) is the major enzyme involved in cholesterol esterification in intestinal mucosa (23).

The purpose of this study was to investigate the physiological regulation of HMG-CoA reductase, MVAPP decarboxylase and ACAT in chick duodenum, jejunum and ileum in order to define the role of each intestinal segment in cholesterol metabolism. Given the fact that isolated epithelial cells have shown higher levels of enzyme activities compared to those found in mucosal scrapings (24), isolated enterocytes were used in the present experiments.

MATERIALS AND METHODS

Materials. Radioactive reagents [3-^{14}C]HMG-CoA, [2-^{3}H]MVA lactone, [2-^{14}C]MVAPP (ammonium salt), [1-^{14}C]oleoyl-CoA and [6-^{3}H]thymidine, were supplied by Amersham International (Amersham, United Kingdom). [1,2,3,6,7,-^{3}H]Cholesteryl oleate was purchased from New England Nuclear (Boston, MA). Radioactive chemicals were diluted with pure unlabelled compounds to obtain the required specific activities. Nonradioactive reagents were from Sigma Chemical Co. (St. Louis, MO), Boehringer Mannheim (Mannheim, Germany) and E. Merck (Darmstadt, Germany).

Animals and diets. White Leghorn male chicks (*Gallus domesticus*), 35-days-old, were used. Newborn animals were obtained from a commercial hatchery and fed *ad libitum* a commercial diet (Sanders A-00, Granada, Spain) which contained (w/w) 45.2% carbohydrate (mainly starch), 3.5% fat and 20.5% protein. Chicks were maintained in a chamber with a light cycle from 0900 to 2100 h and a controlled temperature (28°C). When required, cholesterol (5%, w/w) was added to the commercial diet and given to the animals for 5 d. Food consumption was similar in the three groups of chicks.

Isolation of epithelial cells. Chicks were killed by decapitation (at 2 pm). Intestines were removed, chilled and then divided into three segments designated as duodenum (between gizzard and bile-duct junction), jejunum (a 12-cm region from 15 cm below the bile-duct junction) and ileum (a 12-cm region proximal to the ileo-caecal junction). Isolated epithelial cells from different locations

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; EDTA, ethylenediaminetetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVAPP, mevalonate 5-pyrophosphate; PBS, phosphate-buffered saline.

along the villus-crypt axis were prepared according to Weiser (25) with the modifications suggested by Raul *et al.* (26) consisting of the eversion of the intestinal fragment before incubation in the chelation buffer.

The intestinal segments were immersed in Buffer A (pH 7.3) containing 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 11.1 mM glucose and 1 mM dithiothreitol for 15 min and subsequently in Buffer B (pH 7.2) containing 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 11.1 mM glucose and 1 mM dithiothreitol for a series of 10-min intervals. After collection of the cell fractions in 15 mL of Buffer B in plastic tubes, they were immediately pelleted at 500 $\times$ *g* for 5 min, resuspended in phosphate-buffered saline solution (PBS) and kept on ice until the final fraction was recovered. Aliquots were stained with 0.05% trypan blue in PBS. Cells excluding the dye were considered viable (90–95%). Other details of enterocyte isolation have been reported previously (24,27). Protein contents were determined by the method of Lowry *et al.* (28) with bovine albumin as standard.

Assay of enzyme activities. HMG-CoA reductase activity was measured essentially as described by Shapiro *et al.* (29) with the modifications described by Alejandre *et al.* (30) for neonatal chicks. MVAPP decarboxylase activity was assayed using [2-^{14}C]MVAPP as substrate as previously described (31,32). ACAT was measured as described by Goodman *et al.* (33) with the modifications described by Iglesias *et al.* (34) for chick enterocytes. Acyl-CoA hydrolase was measured using the same conditions as those employed for ACAT, but after chromatography the band of silica gel G containing free fatty acids was directly scraped into counting vials containing 10 mL of the same scintillation liquid used for transferase determination (34).

Determination of free and esterified cholesterol. Lipids were extracted with chloroform/methanol (2:1, vol/vol) as described by Folch *et al.* (35). Total and free cholesterol contents were determined by enzymic colorimetric methods using "Test-Combination Cholesterol" or "Test-Combination Free Cholesterol," respectively, from Boehringer.

RESULTS

Initial studies were undertaken to determine the effects of both cholesterol and cholestyramine feeding on the major regulatory enzymes of cholesterol biosynthesis in isolated enterocytes obtained from chick duodenum, jejunum and ileum. The different diets were used with the idea of affecting the flux of sterol across the intestinal mucosa. As shown in Table 1, cholesterol feeding decreased HMG-CoA reductase activity considerably in all the intestinal regions studied, but inhibition was maximal in ileum where activity in cholesterol fed animals was approximately 30% of that of controls. Cholestyramine feeding increased reductase activity in all the intestinal segments, but the effect was greater in the enterocytes isolated from duodenum. It is important to note that maximal specific activity in controls was observed in jejunum, a region in which the changes induced by cholesterol or cholestyramine feeding were the smallest.

The effects of the same dietary treatments on MVAPP decarboxylase are shown in Table 2. Cholesterol supplementation to the diet induced a significant decrease in decarboxylase activity. However, differences in values for duodenum, jejunum and ileum were less pronounced than those observed for HMG-CoA reductase. Likewise, the effects found after cholestyramine feeding were similar to those found in reductase activity in duodenum. No significant changes occurred in jejunum and ileum.

We have also studied the effect of different diets on cholesterol esterification in chick enterocytes. Table 3 shows that cholesterol feeding markedly increased ACAT activity, the increase being similar in all the intestinal regions assayed. However, the effects of cholestyramine feeding on ACAT activity were less clear. As can be seen in Table 4, no significant differences were found in acyl-CoA hydrolase activity after any of the dietary treatments.

With regard to the cholesterol content of enterocytes isolated from the different regions, cholesterol feeding resulted in enhanced contents of total cholesterol in duodenum and jejunum, while in ileum this increase was smaller (Table 5). In contrast, decreasing the sterol flux by cholestyramine feeding had practically no effect on the

TABLE 1

Effects of Cholesterol and Cholestyramine Feeding on 3-Hydroxy-3-Methylglutaryl-CoA Reductase Activity in Isolated Enterocytes from Chick Duodenum, Jejunum and Ileum[a]

Intestinal segment	Control (C)	Cholesterol (Ch)	Ch/C[b] ratio	Cholestyramine (Ce)	Ce/C[c] ratio
Duodenum	88.85 ± 4.27	48.27 ± 3.86[d]	0.54	321.47 ± 10.40[d]	3.62
Jejunum	149.62 ± 9.40	90.40 ± 5.72[e]	0.60	287.80 ± 7.66[d]	1.92
Ileum	85.80 ± 4.97	28.50 ± 1.49[d]	0.33	184.40 ± 8.77[d]	2.15

[a] Results are expressed as mean values ± SEM of the specific activity (pmol/min/mg protein) obtained in three experiments carried out with enterocytes isolated by the method of Weiser (Ref. 25) from duodenum, jejunum and ileum of three animals. Cells from the same location were pooled. Three determinations were made in each cell pool.

[b] Cholesterol fed/control.

[c] Cholestyramine fed/control.

[d,e] Statistical significance is indicated by [d] $P < 0.001$ or [e] $P < 0.005$ for the effect of the different dietary treatments.

TABLE 2

Effects of Cholesterol and Cholestyramine Feeding on Mevalonate 5-Pyrophosphate Decarboxylase Activity in Isolated Enterocytes from Chick Duodenum, Jejunum and Ileum[a]

Intestinal segment	Control (C)	Cholesterol (Ch)	Ch/C[b] ratio	Cholestyramine (Ce)	Ce/C[c] ratio
Duodenum	98.05 ± 1.39	59.58 ± 3.14[d]	0.61	192.05 ± 9.98[d]	1.96
Jejunum	62.57 ± 3.56	29.25 ± 0.82[d]	0.47	68.10 ± 1.99	1.09
Ileum	63.07 ± 4.48	36.70 ± 2.28[e]	0.58	75.45 ± 3.05	1.20

[a]Results are expressed as mean values ± SEM of the specific activity (pmol/min/mg protein) obtained in three experiments carried out with enterocytes isolated by the method of Weiser (Ref. 25) from duodenum, jejunum and ileum of three animals. Cells from the same location were pooled. Three determinations were made in each cell pool.

[b]Cholesterol fed/control.

[c]Cholestyramine fed/control.

[d,e]Statistical significance is indicated by $^{d}P < 0.001$ or $^{e}P < 0.005$ for the effect of the different dietary treatments.

TABLE 3

Effects of Cholesterol and Cholestyramine Feeding on Acyl-CoA:Cholesterol Acyltransferase Activity in Isolated Enterocytes from Chick Duodenum, Jejunum and Ileum[a]

Intestinal segment	Control (C)	Cholesterol (Ch)	Ch/C[b] ratio	Cholestyramine (Ce)	Ce/C[c] ratio
Duodenum	215.45 ± 3.28	544.12 ± 27.65[d]	2.53	129.42 ± 2.57[d]	0.60
Jejunum	170.62 ± 3.15	487.40 ± 23.00[d]	2.86	145.57 ± 3.14[e]	0.85
Ileum	172.47 ± 10.36	470.05 ± 9.44[d]	2.73	254.00 ± 7.81[d]	1.47

[a]Results are expressed as mean values ± SEM of the specific activity (pmol/min/mg protein) obtained in three experiments carried out with enterocytes isolated by the method of Weiser (Ref. 25) from duodenum, jejunum and ileum of three animals. Cells from the same location were pooled. Three determinations were made in each cell pool.

[b]Cholesterol fed/control.

[c]Cholestyramine fed/control.

[d,e]Statistical significance is indicated by $^{d}P < 0.001$ or $^{e}P < 0.005$ for the effect of the different dietary treatments.

TABLE 4

Effects of Cholesterol and Cholestyramine Feeding on Acyl-CoA Hydrolase Activity in Isolated Enterocytes from Chick Duodenum, Jejunum and Ileum[a]

Intestinal segment	Control (C)	Cholesterol (Ch)	Ch/C[b] ratio	Cholestyramine (Ce)	Ce/C[c] ratio
Duodenum	9.13 ± 0.24	7.85 ± 0.73	0.86	9.69 ± 0.35	1.06
Jejunum	7.92 ± 0.28	7.44 ± 0.42	0.94	6.94 ± 0.48	0.88
Ileum	6.67 ± 0.37	7.01 ± 0.34	1.05	9.15 ± 0.32[d]	1.37

[a]Results are expressed as mean values ± SEM of the specific activity (nmol/min/mg protein) obtained in three experiments carried out with enterocytes isolated by the method of Weiser (Ref. 25) from duodenum, jejunum and ileum of three animals. Cells from the same location were pooled. Three determinations were made in each cell pool.

[b]Cholesterol fed/control.

[c]Cholestyramine fed/control.

[d]Statistical significance is indicated by $P < 0.005$ for the effect of the different dietary treatments.

TABLE 5

Effects of Cholesterol and Cholestyramine Feeding on Cholesterol Content in Isolated Enterocytes from Chick Duodenum, Jejunum and Ileum[a]

Intestinal segment	Control (C)	Cholesterol (Ch)	Ch/C[b] ratio	Cholestyramine (Ce)	Ce/C[c] ratio
Duodenum	22.22 ± 0.54	50.10 ± 1.85[d]	2.25	20.85 ± 0.78	0.94
Jejunum	25.97 ± 0.73	44.90 ± 1.18[d]	1.73	27.40 ± 0.57	1.06
Ileum	20.67 ± 0.32	26.30 ± 0.96[e]	1.27	19.85 ± 0.59	0.96

[a]Results are expressed as mean values ± SEM of the specific activity (μg/mg protein) obtained in three experiments carried out with enterocytes isolated by the method of Weiser (Ref. 25) from duodenum, jejunum and ileum of three animals. Cells from the same location were pooled. Three determinations were made in each cell pool.

[b]Cholesterol fed/control.

[c]Cholestyramine fed/control.

[d,e]Statistical significance is indicated by $^{d}P < 0.001$ or $^{e}P < 0.005$ for the effect of the different dietary treatments.

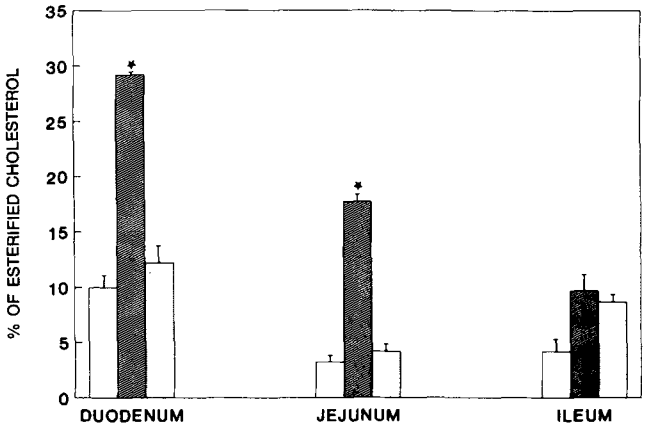

FIG. 1. Effects of cholesterol and cholestyramine feeding on percentage of esterified cholesterol in isolated enterocytes from chick duodenum, jejunum and ileum. Each bar represents the percentage of esterified cholesterol in enterocytes from control (open bars), cholesterol fed (hatched bars) and cholestyramine fed (dotted bars) chicks. Results are expressed as mean values ± SEM of three experiments carried out with pools of three animals. Three determinations were made in each cell pool. Statistical significance is indicated by $*P < 0.001$ for the effect of the different dietary treatments.

cellular total cholesterol content. As can be seen in Figure 1, the changes observed after cholesterol feeding were mainly due to an increase in the percentage of esterified cholesterol in all the intestinal regions assayed.

DISCUSSION

There is still an ongoing debate as to the role of dietary cholesterol in the regulation of cholesterol biosynthesis in intestine. The reasons for the differences between hepatic and intestinal cholesterol metabolism are not entirely clear, although the modes of delivery of cholesterol to the two organs are considerably different (1). Various recent studies suggest that if sufficient cholesterol enters and is accumulated in the intestinal cells, down-regulation of cholesterol synthesis occurs. Our results are in agreement with this fact and with the inhibition of HMG-CoA reductase (9) and MVAPP decarboxylase (21) from chick duodenal mucosa observed after cholesterol feeding. The observed inhibition of decarboxylase due to cholesterol feeding may suggest that a secondary site of regulation does exist at the level of MVAPP decarboxylation.

The location of cholesterol synthesis along the small intestine appears to be dependent on the species that is being studied (1). In the rat, the rate of cholesterol biosynthesis is highest in the duodenum proximal to the ampulla of Vater and in the distal region of the ileum (36,37). Dietschy and Gamel (38) found threefold higher rates of cholesterol synthesis in the human ileum as compared to the rates observed in the duodenum and jejunum. In contrast, the activity of HMG-CoA reductase was shown to be higher in the rabbit jejunum than the activity observed in the ileum (7). Our results are in agreement with those observed on the rabbit, with HMG-CoA reductase activity also being maximal in jejunum.

In contrast to the sterol synthetic enzymes, ACAT activity from chick enterocytes was markedly increased after cholesterol feeding. Our results are in agreement with the fact that this enzyme activity was enhanced after feeding a diet rich in cholesterol to rats (3,39), guinea pigs (40,41) and rabbits (42). The most likely mechanism of stimulation of enzyme activity is an increase in the supply of substrate to an enzyme that is not saturated (23). The finding that the percentage of esterified cholesterol into the chick enterocytes increased after cholesterol feeding (Fig. 1) would support this.

Cholestyramine feeding produced effects of a similar nature on both the HMG-CoA reductase and the MVAPP decarboxylase from chick duodenum. The marked increase in HMG-CoA reductase activity would suggest a decreased cholesterol uptake given the fact that cholesterol levels remain practically unaltered in cells from the cholestyramine fed animals.

On the other hand, the small differences found in acyl-CoA hydrolase activity after cholesterol or cholestyramine feeding suggest that differences in ACAT activity found after the same dietary treatments cannot be due to a different availability of acyl-CoA as exogenous substrate for transferase or to an inhibitory effect on this enzyme due to the presence of free fatty acids (33).

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Radioprotection of Mice by Dietary Squalene

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Male C3H mice were fed a diet containing 2% squalene for 14 d prior to and 30 d subsequent to exposure to 6, 7 or 8 Gy of whole body γ-irradiation (Cesium-137). After 14 d on squalene-supplemented diet, plasma and jejunal tissue squalene levels were 2× and 15× that of controls. Seven days after irradiation, total white cell counts and total lymphocyte counts were substantially depressed in a radiation dose-dependent manner. Although counts in the squalene group were consistently (18–119%) higher than those in the corresponding dietary control group, the differences between dietary groups at any single dose were not significant. Nuclear area of villus cells in the jejunum of both dietary groups was significantly reduced (20%) by day 11 post-irradiation but the nuclear area in squalene-fed mice was significantly greater (15%) than in controls, before and after irradiation. There were no differences in body weight as a function of either diet or radiation dose prior to the first observations of animal lethality. Animal survival was decreased from 100 to 0% at 30 d post-irradiation by radiation doses of 6–8 Gy, with the greatest difference between dietary groups being observed at 7 Gy (median survival times of 12 and 16 d for control and squalene groups, respectively). Overall, survival of squalene-fed mice was significantly prolonged compared with control-fed mice ($P = 0.0054$ by censored multiple regression analysis). It is concluded that squalene conferred some cellular and systemic radioprotection to mice receiving these lethal whole-body radiation doses.
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The consequences of exposure to ionizing radiation, and modifications of these effects, are of considerable interest given the ever-increasing radiation exposure to the population as a result of nuclear power production, energy conservation and radionuclide use. It has been suggested that various chemical structures may protect against the acute cell and tissue toxicities and the delayed carcinogenesis that are induced by radiation *via* generation of free radicals and various ionic species. Among the agents with potential ability to protect cells from radiation damage are those which scavenge or stabilize free radicals. One such compound, β-carotene, is known to offer some protection against radiation-induced toxicity and mortality in mice (1–3). Squalene is a 30-carbon chain with 6 double bonds that is structurally similar to β-carotene. Squalene might also reduce radiation-induced damage, based on the observation that squalene exhibits antioxidant activities (4) and stabilizes oxygen radicals (5). In addition, dietary supplementation with squalene might alter cellular response to radiation *via* a mechanism of enhanced cholesterol metabolism pathways (6).

In humans, about 60% of dietary squalene is absorbed (6) with most serum squalene being in association with the very low density lipoproteins (7). A squalene and sterol carrier protein then appears to carry the nonpolar squalene into the aqueous phase of cells where it is deposited and utilized for cholesterol synthesis (8). Distribution of squalene in human tissues is ubiquitous, with greatest concentrations occurring in the skin and adipose tissue. In rats, dietary squalene supplementation results in an activation of the cholesterol synthesis pathways (9). In other reports, 1% squalene in the diet for periods of 10 to 21 d has been shown to increase tissue levels of squalene, specifically in serum, intestinal mucosa, liver and adipose tissue (10). In intestinal mucosa, squalene increased from an average of 11 μmol/kg to over 1100 μmol/kg after 10 d of dietary supplementation (10).

In the experiment described here, the potential radioprotective effect of dietary squalene was investigated in mice whose diet had been supplemented with 2% squalene starting 14 d prior to exposure to whole body irradiation. The endpoints examined included animal body weight, total white blood cell and lymphocyte counts, nuclear area of jejunal villus cells, plasma and intestinal mucosa levels of squalene and animal survival.

MATERIALS AND METHODS

C3H, male mice aged 5 to 6 wks, weighing 20–25 g, were purchased from Charles River Laboratories (Madison, WI). Appropriate guidelines for humane animal use were followed throughout, after approval by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center (Kansas City, KS). Animals were randomly assigned to receive control or squalene-supplemented diets and within each group to be exposed to 6, 7 or 8 Gy of ionizing radiation. Each of the six groups consisted of 15 mice. Two additional groups of six animals each were randomized to the two diet groups but were not irradiated. These provided non-irradiated controls for the jejunal villus cell measurements and tissue squalene levels. Mice were housed in groups of five or six in plastic mouse cages with microisolation covers in a temperature-controlled facility with a 12-h light/dark cycle. For cage changing, mice were transferred to sterile cages under an air flow hood to minimize the transfer of microorganisms from other mice in the facility. With the exception of cage changing, sterile procedures for animal handling were not followed. After a 3-d acclimation period on arrival, mice were fed, *ad libitum* for 14 d, powdered rodent chow No. 5001 (Ralston Purina, St. Louis, MO) with 2% purified squalene (Sigma Corp., St. Louis, MO) or 2% cornstarch added for two weeks prior to irradiation. Following irradiation, the supplement to the rodent chow was continued for the remaining 30 d of the experiment.

The radiation source was a Cesium-137 blood products irradiator (Model 143-45A, J.L. Shepherd Inc., San Fernando, CA) The mice were irradiated in groups of five in a 10-cm diameter stainless-steel canister which was rotated at 6 rpm to produce a homogeneous dose distribution. The dose rate was 6.2 Gy per minute. The doses delivered to the irradiated mice closely approximated 6, 7 or 8 Gy. All irradiated animals, not otherwise scheduled

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for examination, were held for 30 d post-irradiation. The number of surviving mice in each group was recorded daily at 5:00 p.m. All animals were weighed prior to irradiation and then on post-irradiation days 2, 3, 4, 6, 8 and 10. After post-irradiation day 10, survivors were weighed three times per week.

Six mice from each irradiated group were selected for white blood cell count determinations. Blood (1 μL) was taken from the tail (collecting tube applied directly to the cut end of the tail) for the analysis just prior to radiation exposure and then again on post-irradiation day 7. This day was chosen based on other studies of depression of white cell count following irradiation (11). Total white blood cells were counted in a Nebauer hemacytometer using a light microscope at 400× magnification. The differential count for lymphocytes was performed on blood smears from a drop of tail blood stained with Wright's stain.

The six control-fed and six squalene-fed mice in the non-irradiated group were killed after 14 d on the diets for squalene concentration analysis and to serve as controls for jejunal damage in the irradiated mice. Proximal jejunal sections, pooled within diet groups, were frozen by dry ice/acetone immersion. Plasma and jejunal samples were stored at −20°C for later determination of squalene content. Actual concentrations of squalene in the intestinal mucosa and pooled plasma of both groups of non-irradiated mice were determined by gas-liquid chromatography using the method described by Liu *et al.* (7). Pooled jejunal samples, weighing 4 g each in 10 mL of distilled water containing 200 μg of 5α-cholestane as an internal standard, were homogenized using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY). The analytical procedures for squalene determination were identical for plasma and homogenized jejunum. The non-saponifiable lipids were recovered from the extraction mixture with petroleum ether. Squalene concentrations were determined using a gas chromatograph (model GC 14A, Shimadzu, Kyoto, Japan), equipped with a single flame-ionization detector and a glass column (4 × =1/4″ i.d.) packed with a Dexil 100 supelcoport (Supelco, Bellefonte, PA). A Shimadzu integrating unit (Chromatopac CR-601) was used to compute and record data.

Six survivors from each irradiated group were randomly selected, anesthetized with ether and decapitated on post-irradiation day 11 for study of gastrointestinal damage. This day was chosen as the time when half the animals in the most affected group (8 Gy, control diet) had died. A standard section of proximal jejunum was dissected from each animal and rinsed of residual luminal contents, using phosphate buffered saline. After 4 d in neutral buffered formalin, the jejunal cross sections were processed for histological examination of 2 μm thick plastic sections (L.R. White; Ernest F. Fullam, Inc., Latham, NY). To evaluate intestinal injury, the area of the cross section of nuclei of villus cells was measured using the Zidas digitizing pad at 400× magnification with a Zeiss microscope (Carl Zeiss, Thornwood, NY). The nuclear areas of 10 cells located on a single villus were determined for each intestinal section. A representative villus was chosen, with no areas of significant necrosis or hemorrhages, in which the nuclei of the villus cells were clearly identifiable. Large nuclei at the tips of the villus were measured in cross section to obtain the maximum area through the center of the nucleus.

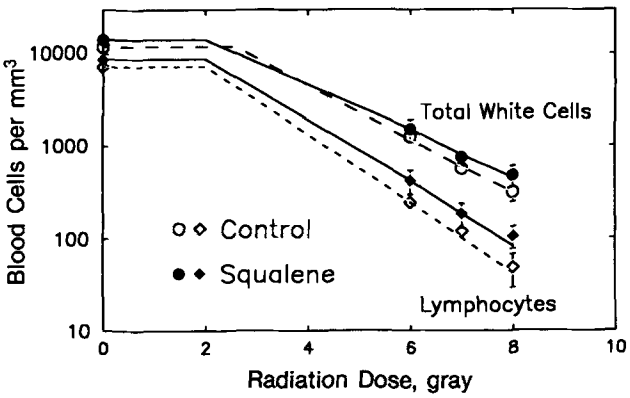

FIG. 1. Total white blood cell counts (circles) and total lymphocyte counts (diamonds) seven days after radiation exposure to control (open symbols) and squalene-treated (closed symbols) animals. The error bars indicate standard errors.

For comparison of post-irradiation survival between groups, a Gehan-Wilcoxon test for single-censored samples was performed. To test for the effect of radiation dose and diet supplementation, a censored multiple regression analysis was conducted using the survival analysis package of the Number Cruncher Statistical Software program (J.L. Hintze, Kaysville, UT). The data for total white blood cell counts, differential lymphocyte counts, body weights and villus nuclear areas all exhibited normal distributions and were analyzed by Analysis of Variance. Blood cell counts were also subjected to linear regression analysis.

RESULTS

Tissue squalene concentrations. Concentrations of squalene in pooled samples of plasma and jejunal tissue of twelve non-irradiated mice, six fed the control diet and six fed the squalene-supplemented diet, were determined. The pooled plasma concentration of squalene in mice maintained on the diet supplemented with squalene was 9.2 mg/dL, over twice the level of the squalene found in the pooled plasma of control animals (4.4 mg/dL). Pooled jejunal squalene concentrations in mice supplemented with squalene exceeded those of control-fed mice by more than 15-fold (squalene diet: 470 μg/g tissue weight, control diet: 30 μg/g).

White blood cell counts. Prior to irradiation, the total white blood cell counts of squalene-supplemented mice were 18% higher than those from control mice, although this was not significantly different (Analysis of Variance, $P = 0.21$). Seven days after irradiation, total white blood cell counts had dropped to 10% or less of control, pre-irradiation levels for all radiation doses ($P < 0.0003$). Squalene-treated mice had white blood cell counts 21–52% greater than did control mice at the same radiation doses, although these differences were again not statistically significant. While the reduction of total white blood cell count occurred in a log-linear fashion with increasing radiation dose (Fig. 1), there was no difference in the slopes of the regression lines between control-fed and squalene-fed groups.

TABLE 1

Body Weights (g, mean ± SE) as a Function of Time Post-Irradiation

Dose:	Treatment groups					
	6 Gy		7 Gy		8 Gy	
Diet:	Control	Squalene	Control	Squalene	Control	Squalene
Day						
0	22.5 ± 0.3	22.3 ± 0.2	22.9 ± 0.3	22.6 ± 0.5	23.1 ± 0.4	22.7 ± 0.3
n[a]	15	15	15	15	15	15
6	22.9 ± 0.2	22.6 ± 0.2	22.9 ± 0.3	22.5 ± 0.5	23.2 ± 0.4	22.5 ± 0.4
n	15	15	15	15	15	15
10	24.0 ± 0.3	23.2 ± 0.4	23.3 ± 0.3	23.0 ± 0.5	23.0 ± 0.5	22.7 ± 0.5
n	15	15	13	14	12	14
16	23.3 ± 0.6	22.8 ± 0.3	D[b]	18.7 ± 1.7[c]	D	D
n	8	9	0	5	0	0
20	25.0 ± 0.5	23.9 ± 0.6	D	19.6 ± 2.2	D	D
n	7	9	0	2	0	0

[a]Number of animals contributing to the values.
[b]D, all animals in the group died from radiation.
[c]On day 16, squalene-treated mice receiving 7 Gy had significantly lower body weights than control or squalene-treated mice receiving 6 Gy ($P < 0.05$).

TABLE 2

Nuclear Area of Villus Cells (μm^2 ± SE)[a] in Mice Exposed to Graded Doses of Radiation and Maintained on Squalene-Supplemented or Control Diets

Dose	Diet	
	Control	Squalene
0 Gy	14.85 ± 0.62	17.14 ± 1.00[b]
6 Gy	11.77 ± 0.48[c]	14.53 ± 0.45[b,c]
7 Gy	11.77 ± 0.33[c]	12.63 ± 0.45[c]
8 Gy	11.90 ± 0.52[c]	14.94 ± 0.66[b,c]

[a]The 160 nuclear measurements from six mice per group, with the exception of the control group exposed to 8 Gy, which had only five mice due to one early death.
[b]Significant difference between control and squalene-treated group (Analysis of Variance, $P < 0.05$).
[c]Significantly different from respective non-irradiated groups (0 Gy) (Analysis of Variance, $P = 0.05$).

Similarly, total lymphocyte counts prior to irradiation were 20% higher in squalene-supplemented mice than in control mice although the difference was not significant ($P = 0.14$). Seven days after exposure to ionizing radiation, total lymphocyte counts were profoundly lower in all animals (Fig. 1). Squalene-treated mice had counts 55-119% greater than did control mice at the same radiation doses (not statistically significant). Degree of lymphocytopenia again correlated with radiation dose delivered, with lower counts occurring following higher doses, but the slopes of the regression lines were not significantly different.

Thus, for both total white blood cell count and total lymphocyte count, there was a log-linear decrease as a function of radiation dose received, but no statistically significant differences between blood counts from control diet *vs.* squalene-fed mice. There was also no relationship between animal survival and individual blood counts, or between those animals selected for blood drawing and those not.

Body weights. Weights of control and squalene-supplemented mice exposed to 6 Gy or 7 Gy were compared on post-irradiation days 0, 6, 10, 16 and 20 (Table 1). On post-irradiation days 0, 6, 10, and 20, body weights of mice exposed to 6 or 7 Gy were not significantly different. However, on post-irradiation day 16, weights of squalene-treated mice exposed to 7 Gy were significantly lower than weights of control or squalene-treated mice exposed to 6 Gy ($P < 0.05$). At day 16 post-irradiation, all animals exposed to 8 Gy and most control animals exposed to 7 Gy were dead and thus could not be included in the statistical analysis of weights (Table 1).

Nuclear area of villus cells. The nuclear areas in cross sections of cells located at the tips of villi in the proximal jejunum were determined from histological preparations. In both squalene-treated and control mice, the villus nuclear area was significantly greater in animals not exposed to radiation than in animals exposed to 6, 7 or 8 Gy ($P < 0.05$) (Table 2). However, in both control and squalene-treated mice, no significant dose-related differences in nuclear area were found. Among non-irradiated diet groups, nuclear areas were greater in the squalene-supplemented mice than controls; similarly among irradiated mice, groups supplemented with squalene exhibited greater nuclear areas than did control-fed groups ($P = 0.02$). Post-irradiation nuclear areas of squalene-fed mice approximated those of non-irradiated mice on the control diet.

Survival. The mortality data obtained through post-irradiation day 30 for control and squalene-supplemented mice are presented in Figure 2. Survival times were compared for the animals in each diet/dose group (Table 3). With increasing radiation dose, the survival times of animals decreased in both control and squalene-supplemented mice. The effect of irradiation in both diet groups was marked, with the median survival time following 8 Gy less than half that for 6 Gy. In both diet groups, the survival following 6 Gy was statistically significantly different ($P < 0.002$ by Gehan-Wilcoxon analysis) than after either 7 or 8 Gy. Overall, increasing radiation dose was a significant ($P < 0.0001$ by censored multiple regression analysis) determinant of shorter survival time.

The difference in survival between squalene-supplemented mice and controls was greatest at the radiation dose of 7 Gy (median survival times of 16 *vs.* 12 d).

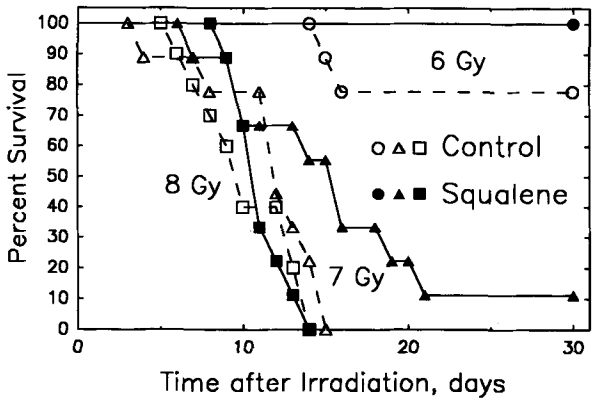

FIG. 2. Survival as a function of post-irradiation time and radiation dose (6 Gy, ○, ●; 7 Gy, △, ▲; 8 Gy, □, ■) for mice on control (open symbols) or squalene-supplemented diets (closed symbols).

TABLE 3

Combined Effects of Diet and Radiation Dose on Survival Post-Irradiation

	Median survival time (range) in days[a]	
Radiation dose	Control diet	Squalene diet
6 Gy	>30 (15-30+)	<30 (30+)
7 Gy	12 (4-15)	16 (7-30+)
8 Gy	11 (6-14)	12 (9-14)

[a]30+ Indicates animals sacrificed at 30 d post-irradiation.

At 6 and 8 Gy, there was no difference in survival between the groups because there was minimal and total lethality, respectively. None of the comparisons between control and squalene diet at individual radiation dose levels were statistically significant by the Gehan-Wilcoxon analysis. However, in a global analysis, supplementation with squalene was identified as a significant ($P = 0.0054$ by censored multiple regression analysis) determinant of greater survival time.

DISCUSSION

The survival time of an animal following exposure to ionizing radiation is related to the primary target tissues affected. The causes of radiation-induced lethality are generally categorized as central nervous system, gastrointestinal or hematopoietic syndromes. Of the animals exposed to radiation doses of 6, 7 or 8 Gy, most deaths occurred after 7 d in both diet groups, implicating death due to the hematopoietic syndrome. A dose-dependent decrease in survival time was observed overall, as well as individually for both diet groups. The greater survival among the squalene-supplemented mice compared with controls demonstrates a protective effect of squalene against lethal radiation-induced injury. Control and squalene-fed animals exposed to 6 Gy had similar survival times, because there was only minimal lethality produced by this dose. Conversely, control and squalene-fed animals exposed to 8 Gy also had similar survival times, reflecting an inability of squalene to prevent or delay death at this lethal radiation dose. The survival rates achieved in the present investigation were similar to those reported for CBA mice given β-carotene (90 mg/kg diet) immediately after exposure to 6 or 7 Gy (3). In that study, β-carotene was shown to prolong survival after radiation.

Leukopenia was observed in all irradiated animals 7 d after radiation exposure. Although not statistically significant, the total white blood cell and lymphocyte counts were consistently higher in the squalene-treated mice. A similar finding of moderation of radiation-induced leukopenia was been reported (1) for C57 mice maintained on diets supplemented with 90 mg β-carotene per kg diet and exposed to 6 Gy of whole body irradiation. The protective effect of dietary squalene on leukopenia in the present study was less pronounced than the protective effect reported for β-carotene. One mechanism suggested for protection from radiation-induced leukopenia was a stimulation of the immune system (12,13). An alternative suggestion is that β-carotene can scavenge free radicals (14) and a similar mechanism (4) may account for the effects of squalene observed here.

After the two-week feeding period, pooled plasma and jejunal squalene concentrations were greater in squalene-fed mice than controls, as reflected by a twofold greater level in blood and an over 15-fold greater level in intestine in the squalene-fed animals. The greater tissue squalene concentrations were expected since squalene supplementation with 1% squalene for 10 d in other studies also resulted in tissue increases (10,15). In view of the greater squalene levels in non-irradiated, squalene-fed mice, it is expected that the irradiated squalene-supplemented mice would have had similar tissue squalene levels, especially at the time of irradiation (14 d after initiation of squalene diet).

Body weight loss and general wasting are important signs of radiation-induced toxicity (3). Based on the times of death in the present investigation, the animals succumbed due to the hematopoietic syndrome and not as a result of gastrointestinal injury. This may explain the absence of weight loss in mice until 16 d post-irradiation. Also, once the smallest (weakest) animals died and were no longer included, the mean weight of the group would increase. Overall, no protection for weight loss was afforded by dietary squalene.

The nuclear area of villus cells in the jejunum of squalene-supplemented mice exceeded the nuclear areas of control-fed mice in both irradiated and non-irradiated groups. The greater villus nuclear area (most likely associated with an increase in nucleolar size) seen in animals supplemented with squalene compared to control-fed animals suggests an enhancement of cellular synthetic rates by squalene. This increased synthetic activity may be specifically related to increased cholesterol synthesis which has been shown to result from increased plasma levels of squalene (16), or may be a general metabolic effect. A low concentration of peroxidized squalene, such as might be present after irradiation, has been shown to increase incorporation of amino acids (17). Other conditions that give rise to increases in nuclear area include normal synthetic activity (18,19) and synthesis prior to cell division (20). The positive relationship observed here for survival and nuclear area between diet groups supports the speculation that squalene might exert an enhancing effect on cellular metabolic processes that would ameliorate the damaging effects of radiation.

The data obtained from this research support the conclusion that dietary squalene provides some protection from the adverse effects of whole-body ionizing radiation. This is expressed at several levels: jejunal villus cell morphology, white blood cell counts and animal survival. However, the latter is demonstrated only at a specific dose, with the survival-prolonging protective effects of squalene greater in mice exposed to a dose of 7 Gy than in mice exposed to 6 or 8 Gy. The mechanisms by which squalene could protect against radiation-induced damage remain speculative, but include scavenging of free radicals or reactive oxygen species, stimulating the immune response, protecting cellular organelles or improving cellular repair responses, or simply stimulating cell proliferation. Whether the high levels of squalene in certain diets already contribute to a protection against radiation-induced carcinogenesis, or whether dietary supplementation of squalene might have potential use in radiation therapy of malignancies, remains even more speculative.

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METHOD

2-Alkenyl-4,4-dimethyloxazolines as Derivatives for the Structural Elucidation of Isomeric Unsaturated Fatty Acids

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Several types of unsaturated fatty acid methyl esters were converted into 4,4-dimethyloxazoline (DMOX) derivatives and analyzed by mass spectrometry to further evaluate the feasibility of using this derivative for locating the positions of double bonds in isomeric fatty acids. Five isomeric 20-carbon tetraenoic acids were analyzed in which the four *cis* double bonds were systematically moved from the 4,7,10,13- to the 8,11,14,17-positions. It was possible to locate the positions of all four double bonds in the 7,10,13,16- and 8,11,14,17-isomers by appropriate ions differing by 12 atomic mass units. In a similar way the three terminal double bonds in the 4,7,10,13-, 5,8,11,14- and 6,9,12,15-isomers could be assigned. Odd-numbered ions at *m/z* 139, 153 and 167 which are accompanied by an even mass ion at 138, 152 and 166, respectively, are diagnostic for DMOX derivatives of acids with their first double bond, respectively, at positions 4, 5 and 6. It was thus possible to assign the location of all four double bonds in these three isomers. A comparison of the spectra of the DMOX derivatives of 17,17,18,18-d_4 *vs.* 9,10,12,13-d_4 linoleic acid suggests that double bonds preferentially migrate toward the polar end of the molecule prior to fragmentation. The merit of using DMOX derivatives to locate double-bond positions in mono- and dicarboxylic acids, produced during β-oxidation of polyunsaturated fatty acids, was evaluated. The spectra of 3-*cis*- and 4-*cis*-decenoic acids differ as do the spectra of 8-carbon dicarboxylic acids with their double bonds at positions 3 and 4.
Lipids 28, 561–564 (1993).

Several techniques have been used to locate the positions of double bonds in polyunsaturated fatty acids. Unsaturated acids may be treated with ozone, and the resulting ozonide may be either reduced or oxidized followed by characterization of the resulting fragments (1). Unsaturated fatty acids may also be cleaved, at the double bond, into short-chain mono- and dicarboxylic acids by treatment with periodate-permanganate (2). Alternatively, double bonds may be converted to osmate esters which, upon reduction, yield diols. Following silyation they are analyzed by mass spectrometry. The fragments produced by cleavage between adjacent trimethylsilyl groups determine the position of the double bonds in the original molecule (3,4).

With the above methods it is often difficult to isolate and characterize all of the fragments produced when degradative techniques are used. The osmium technique determines the sites of unsaturation in the intact molecule, but the resulting silyl derivatives of polyunsaturated acids have long retention times when analyzed by gas chromatography. In addition, many of the smaller mass spectrometers have upper mass limits too low to detect all the fragments produced from polyunsaturated fatty acids containing multiple sites of unsaturation.

A third general method involves derivatizing fatty acids with agents that enhance charge stabilization to minimize double-bond migration. A number of reagents including pyrrolidine (4,5), 3-pyridylcarbinol (4,6) and 2-amino-2-methylpropanol (AMP) (7–11) have been employed as derivatizing agents. The 4,4-dimethyloxazoline (DMOX) derivatives, prepared by reacting either fatty acids (7) or methyl esters (8) with AMP have good chromatographic properties and in general provide definitive information about double bond positions.

In the study reported here, we further evaluated the limitations of the current methodology by comparing the mass spectra of five isomeric 20-carbon acids in which the four *cis*-double bonds were moved from the 4,7,10,13- to the 8,11,14,17-position. The spectra of the DMOX derivatives of 17,17,18,18-d_4 *vs.* 9,10,12,13-d_4 linoleic acid were compared to further determine the mechanism of fragmentation with this derivative. The localization of double bonds in short-chain mono- and dicarboxylic acids produced during the abnormal β-oxidation of unsaturated fatty acids is a difficult problem due to the relatively small amounts of materials produced (12). In the present study we have shown that the spectra of DMOX derivatives of 3-*cis*- *vs.* 4-*cis*-10:1 as well as of 3-*cis*- and 4-*cis*-1,8-octenedioic acids differ sufficiently to readily distinguish these isomeric compounds.

MATERIALS AND METHODS

Materials. 2-Amino-2-methylpropanol was obtained from MTM Research Chemicals (Windham, NH). The methyl esters of 4,7,10,13-, 6,9,12,15-, 7,10,13,16- and 8,11,14,17-eicosatetraenoic acids were made by total synthesis (13). Methyl 5,8,11,14-eicosatetraenoate was from Nu-Chek-Prep (Elysian, MN). Linoleic acid, labeled with deuterium at positions 9,10,12 and 13, was made by reducing 9,12-octadecadiynoic acid with deuterium gas in the presence of Lindlar's catalyst. In order to prepare 17,17,18,18-d_4 linoleic acid, the hydroxyl group of 4-pentyn-1-ol (MTM Research Chemicals) was blocked by reaction with ethyl vinyl ether (Aldrich Chemical Company, Milwaukee, WI). The triple bond was then reduced with deuterium gas in the presence of Wilkinson's catalyst (14). The blocking group was removed by refluxing with methanol in the presence of sulfuric acid (15). The deuterated 1-pentanol was converted to d_4-pentyl bromide which in turn was used to synthesize 9,12-octadecadiynoic acid-17,17,18,18-d_4 (16). Lindlar's reduction with hydrogen yielded the desired ethylenic acid. To determine the isotopic composition of the deuterated linoleic acids, they were derivatized with *N*-methyl-*N*-(*t*-butyl-dimethylsilyl)-trifluoracetamide

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Abbreviations: AMP, 2-amino-2-methylpropanol; DMOX, 4,4-dimethyloxazolines; THP, tetrahydropyran.

+ 1% *t*-butyl-dimethylchlorosilane (Regis Chemical Co., Morton Grove, IL) (17). The appropriate ion chromatograms corresponding to the formation of ions at M-57 were then integrated. The isotopic composition in percent of the 9,10,12,13-d_4 and 17,17,18,18-d_4 were d_4, 95.9; d_3, 3.7; d_2, 0.3; d_1, 0.1 and d_4, 94.8; d_3, 3.1; d_2, 2.1; d_1, 0.0, respectively.

4-*cis*-Decenoic acid was prepared by coupling 1-bromopentane with the sodium salt of the tetrahydropyran (THP) derivative of 3-butyn-1-ol in liquid ammonia. The blocking group was removed and the 3-nonyn-1-ol was reduced with Lindlar's catalyst. The 3-*cis*-nonen-1-ol was converted to the mesylate (18) which in turn was reacted with sodium cyanide in dimethyl sulfoxide (19). The resulting nitrile was hydrolyzed to the methyl ester with 25% anhydrous HCl in methanol. To make 3-*cis*-decenoic acid, the sodium salt of the THP derivative of 3-butyn-1-ol was coupled with 1-bromohexane. After cleavage of the blocking group, the alcohol was oxidized to 3-decynoic acid (20) which was then reduced to 3-*cis*-decenoic acid. To make 3-*cis*-1,8-octenedioic acid, the hydroxyl group of 5-hexyn-1-ol was blocked by reaction with dihydropyran. This compound was reacted with sodamide in liquid ammonia followed by addition of ethylene oxide (21). Following cleavage of the blocking group, the 1,8-dihydroxy-3-octyne was oxidized to the acetylenic dicarboxylic acid which in turn was reduced with Lindlar's catalyst to give 3-*cis*-octenedioic acid. To prepare 4-*cis*-octenedioic acid, the sodium salt of the THP derivative of 3-butyn-1-ol was reacted with ethylene oxide in liquid ammonia (21). Following cleavage of the blocking group, the 3-hexyn-1,6-diol was reduced with Lindlar's catalyst. The diol was converted to the dimesylate which in turn was reacted with sodium cyanide in dimethyl sulfoxide. Hydrolysis with 25% anhydrous HCl in methanol yielded dimethyl 4-*cis*-octenedioate.

Analysis of DMOX. The DMOX derivatives were prepared by adding 250 μL of AMP to 500 μg of the fatty acid methyl ester in screw-cap vials. The vials were flushed with N_2, capped and heated at 180°C for 18 h (8). The reaction mixture was cooled and dissolved in 3 mL of dichloromethane which was washed twice with 1 mL of water. After drying the organic phase with anhydrous Na_2SO_4, the dichloromethane was removed under a stream of N_2. The samples were dissolved in *iso*-octane for analysis by gas chromatography/mass spectrometry.

Mass specctrometry was carried out with a Hewlett-Packard model 5970A mass selective detector and a 5790 gas chromatograph (Palo Alto, CA). For the analysis of the five 20-carbon DMOX derivatives, the oven contained a 30 m × 0.25 mm DB-1 capillary column with a film thickness of 0.25 μm (J&W Scientific, Rancho Cordova, CA). All injections were carried out in the splitless mode at 70°C. After 1 min the oven was programmed at 30°C/min to 240°C. All other DMOX derivatives were injected in the split mode at 170°C using a 30 m by 0.25 mm DB-225 capillary column (J&W Scientific) with a film thickness of 0.25 μm. Spectra were recorded at an ionization energy of 70 eV. The Y-axis in all spectra shown represents relative percentage abundance of the ions.

RESULTS AND DISCUSSION

The spectra of the five 20-carbon DMOX derivative all showed major ions at *m/z* 113 and 126 as well as a parent ion at *m/z* 357. The ion at *m/z* 113 is in essence a McLafferty rearrangement ion formed by migration of the γ-hydrogen followed by cleavage between carbon atoms 2 and 3 while the ion at *m/z* 126 is probably a cyclic ion formed by cleavage between carbons 4 and 5 (7). In their analysis of pyrrolidide derivatives of fatty acids, Andersson and Holman (5) observed that the location of double bonds could be determined when an interval of 12 atomic mass units rather than 14 is observed between the most intense peak in a cluster of fragments containing n and n − 1 carbon atoms. The double bond is then located between n and n + 1 carbon atoms in the molecule. The partial mass spectra of the DMOX derivative of 8,11,14,17-20:4 is similar to that published by Yu *et al.* (9) and is shown in Figure 1 along with the spectrum of the 7,10,13,16-20:4 isomer. From these spectra it is clear that all four double bonds in both isomers can be located by the fragmentation pattern. The ions at *m/z* 182 and 194 (Fig. 1A) show that the first double bond is at position 8 in the 8,11,14,17-20:4 isomer (9) while the ions at *m/z* 168 and 180 (Fig. 1B) define that the position of the first double bond of the second isomer is at the 7-position. Double bonds at the other sites were determined in a similar way as shown in Figure 1.

The mass spectrum of the 6,9,12,15-isomer as shown in Figure 2A is similar to that published by Yu *et al.* (9). According to the rule of Andersson and Holman (5), the spectrum should contain an ion at *m/z* 154, which would be formed by cleavage between carbons 5 and 6 while cleavage between carbons 6 and 7 would be expected to produce an ion at *m/z* 166. The spectrum of the DMOX derivative of 6,9,12,15-20:4 had an intense ion at *m/z* 152 as well as ions of about equal intensity at *m/z* 166 and

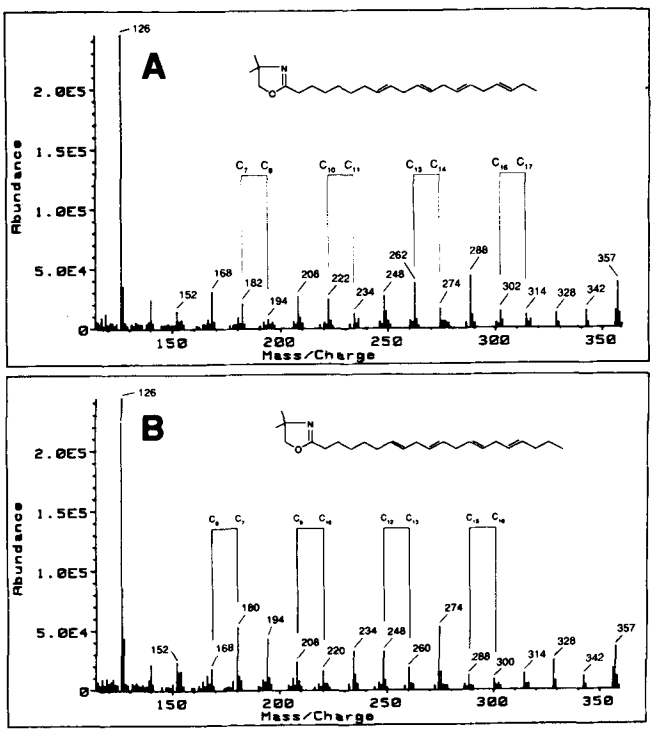

FIG. 1. Mass spectra of the 4,4-dimethyloxazoline derivatives of 8,11,14,17- (A) and 7,10,13,16-eicosatetraenoic acids (B).

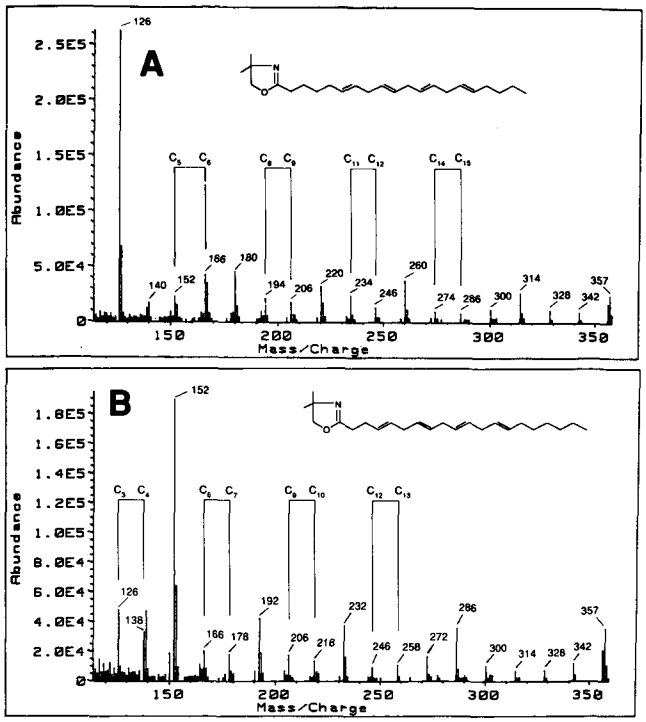

FIG. 2. Mass spectra of the 4,4-dimethyloxazoline derivatives of 6,9,12,15- (A) and 4,7,10,13-eicosatetraenoic acids (B).

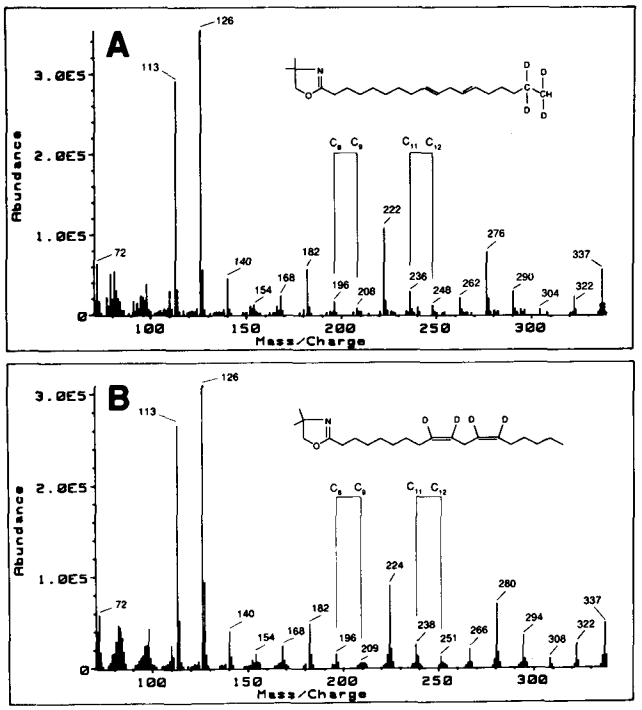

FIG. 3. Mass spectra of the 4,4-dimethyloxazoline derivatives of 17,17,18,18-d_4 (A) and 9,10,12,13-d_4 linoleic acids (B).

167. A similar type of fragmentation pattern has been reported for mono- and polyunsaturated fatty acids with their first double bond at position 6 (7–9). The other three double bonds in the molecule were clearly located by ions which differed by 12 mass units. The spectrum of the DMOX of 5,8,11,14-20:4 was similar to that reported previously in that it had a base peak at *m/z* 113 and an intense odd-numbered ion *m/z* 153 (31% of the base peak) (7). The mechanism for the formation of this ion has not been explained (9). Similar to the DMOX derivative of 4,7,10,13,16,19-22:6 (7), the spectrum of the 4,7,10,13-isomer had an ion at *m/z* 126 and a pair of ions of about equal intensity at *m/z* 138 and 139 (Fig. 2B). Again, the location of the double bonds at positions 7, 10 and 13 could be deduced from the spectra. These results, together with those reported in the literature (7–9), show that the location of all double bonds in a fatty acid can be determined by the interval of 12 mass units between appropriate ion clusters when the first double bond is at position 7 or further down the carbon chain. When the first double bond in the molecule is at carbons 4, 5 or 6, it is possible to locate the position of the three terminal double bonds by the 12 mass unit rule. The location of the first double bond however cannot be defined by an empirical rule. The presence of odd-numbered ions at *m/z* 167, 153 and 139, which are accompanied by *m/z* 166, 152 and 138, respectively, is diagnostic ions in the spectra of DMOX derivatives of acids with their first double bond, respectively, at carbons 6, 5 and 4.

The mass spectra of the DMOX derivative of both 17,17,18,18-d_4 and 9,10,12,13-d_4 linoleic acid had molecular ions at *m/z* 337 (Fig. 3). The introduction of deuterium at carbons 17 and 18 did not alter the ion composition in the low mass region *vs.* that reported for the DMOX derivative of unlabeled linoleate (7). Cleavage between carbons 16 and 17 of both labeled 17,17,18,18-d_4 and unlabeled linoleate (7) gave an ion at *m/z* 304. The presence of an ion at *m/z* 322 in the undeuterated derivative could arise either by cleavage between carbons 17 and 18 or by loss of a methyl group from the oxazoline ring. The presence of an ion at *m/z* 322 in the DMOX derivative of 17,17,18,18-d_4 linoleate (Fig. 3A) shows that this ion is formed by loss of a methyl group from the ring rather than by cleavage between carbons 17 and 18 which would have resulted in the loss of 17 mass units, *i.e.*, loss of $CHD_2^{\bullet}$.

The fragmentation pattern at sites of unsaturation may be visualized as requiring the movement of double bonds one position toward the polar end of the molecule prior to fragmentation (5,9). Cleavage between carbons 9 and 10 of the DMOX derivative of unlabeled or 17,17,18,18-d_4 linoleic acid yields a cluster of small ions, the major one of which is at *m/z* 208 (7). With the 9,10,12,13-d_4 isomer, a small ion peak at *m/z* 209 was detected, but this cluster contained other ions of low intensity (Fig. 3B). Cleavage between carbons 10 and 11 of the 17,17,18,18-d_4 analog yielded an ion at *m/z* 222 *vs.* at 224 for the 9,10,12,13-d_4 isomer thus establishing that carbons 9 and 10 contain deuterium atoms. Cleavage between carbons 12 and 13 of the 17,17,18,18-d_4 linoleate derivative gave an ion at *m/z* 248 which increased to 251 when the acid was labeled with deuterium at the double bonds. Clearly then the major ion formed by cleavage at double bonds can be visualized primarily as requiring the movement of double bonds toward the polar end of the molecule prior to fragmentation as has previously been suggested (5,9).

The DMOX derivatives of both 3- and 4-*cis*-decenoic acids had molecular ions at *m/z* 223 (Fig. 4). The base peak

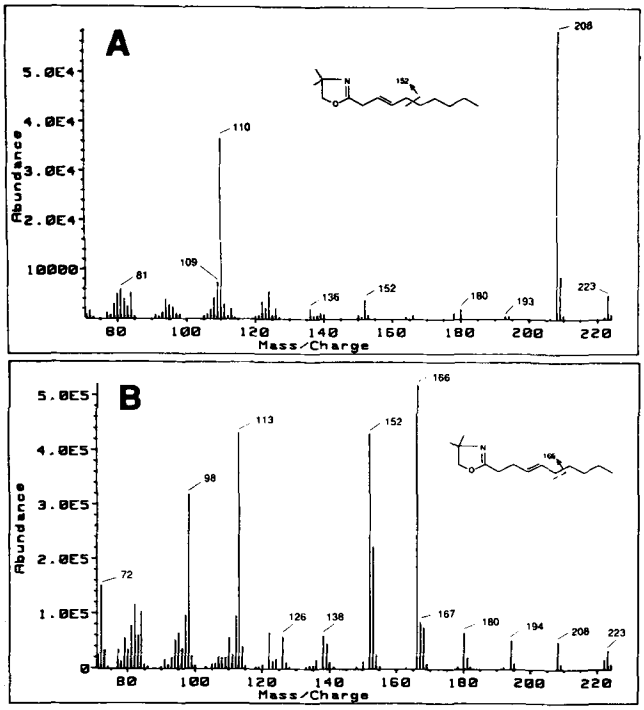

FIG. 4. Mass spectra of the 4,4-dimethyloxazoline derivatives of 3-*cis*- (A) and 4-*cis*-decenoic acids (B).

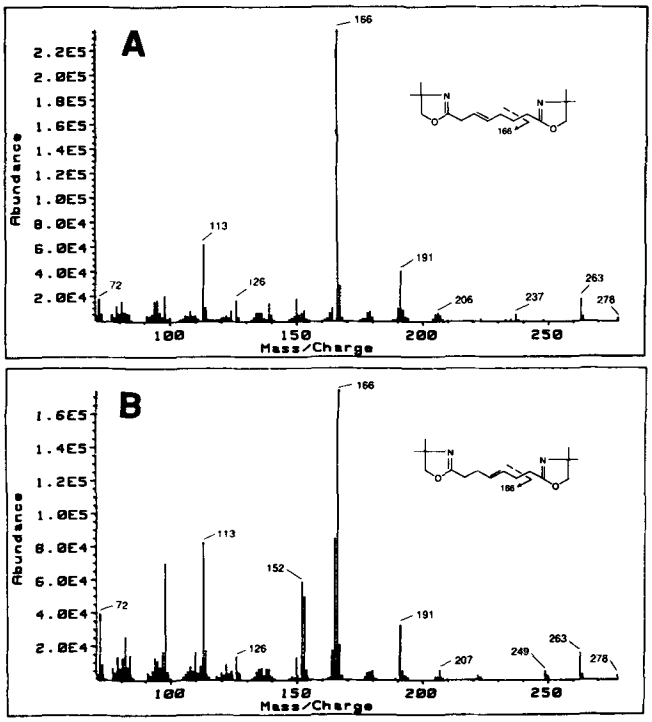

FIG. 5. Mass spectra of the 4,4-dimethyloxazoline derivatives of 3-*cis*- (A) and 4-*cis*-1,8-dicarboxylic acids (B).

for the 3-*cis* isomer was at *m/z* 208 which may be due to the loss of methyl group from the ring. The only other ion of high intensity was one of unknown composition at *m/z* 110. Conversely, the spectrum of 4-*cis* isomer had a base peak at *m/z* 166, and an ion of unknown composition at *m/z* 152 which is characteristic of acids with double bond at position 4 (7-9). The ion at *m/z* 98 corresponds to the fragment formed by the cleavage between carbons 1 and 2 with a positive charge on the heterocyclic ring.

The mass spectra of DMOX derivatives of oct-3-ene-1,8-dicarboxylic acid and oct-4-ene-1,8-dicarboxylic acid are similar in that both had small molecular ion peaks at *m/z* 278 and as well as at 263 (M-15) (Fig. 5). The base peak for both derivatives was at *m/z* 166. The spectrum of the 4-*cis* isomer (Fig. 5B) differs from that of the 3-*cis* isomer in that the ion intensity at *m/z* 113 is almost twice that observed for the 3-*cis* isomer. Most importantly the 4-*cis* isomer had ions at *m/z* 152 and 153 as is observed for other fatty acids with a double bond at position 4 (7-9). The presence of these two ions can readily distinguish the 3-*cis* from the 4-*cis* isomer.

ACKNOWLEDGMENTS

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Acidic Hydrolysis of Plasmalogens Followed by High-Performance Liquid Chromatography

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A simple, quantitative method for determining the plasmalogen content of small samples is reported here. The method uses the different susceptibility to acid-catalyzed hydrolysis of the alkyl, alkenyl and acyl linkages to separate the plasmalogen subclass from the other two non-labile subclasses. Hydrolysis of plasmenylethanolamine and plasmenylcholine was complete after 4 and 1 min of acid treatment, respectively. The acid-catalyzed hydrolysis did not alter the phospholipid fatty acid composition, making this method useful for fatty acid compositional analysis of the plasmalogen subclass. High-performance liquid chromatography was used for separations, and phospholipids were quantitated by assay of lipid phosphorus or by direct quantitation of peak area. Using this method, small amounts (10 nmol) of ethanolamine glycerophospholipid and choline glycerophospholipid are subjected to acid-catalyzed hydrolysis and subsequent separation of the resulting lysocompounds obtained from plasmalogens from the more acid-stable alkylacyl and diacyl glycerophospholipid fractions. Our values for plasmalogens from commercial preparations of choline and ethanolamine glycerophospholipids agree with literature values. The usefulness of the method is demonstrated for small glycerophospholipid samples that are equivalent to samples from cultured neural cells.
Lipids 28, 565–568 (1993).

Plasmalogens are important components of cellular membranes and may be involved in signal transduction following agonist-receptor binding (1,2). Recently a plasmalogen specific phospholipid A_2 has been isolated from bovine brain (3). This enzyme may have an important role in releasing arachidonic acid from the *sn*-2 position of the plasmalogens during receptor mediated events. Plasmalogens are also elevated during and following differentiation of N1E-115 cells suggesting plasmalogens have a role in cellular differentiation (4).

Current methods for the quantitation of ethanolamine and choline plasmalogens are not suitable for the limited sample size obtained from cell cultures. Previous methodology has included iodine addition (5,6), alkaline hydrolysis followed by acid hydrolysis (7), mercuric chloride hydrolysis (8) and acidic hydrolysis (9–13). Most of these methods utilize the acid lability of the alkenyl ether linkage at the *sn*-1 position of the plasmalogens. This bond is quantitatively hydrolyzed by HCl fumes (14). The method described here is a modification of the Horrocks and Sun (15) method replacing two-dimensional thin-layer chromatography (TLC) with two high-performance liquid chromatography (HPLC) separations. This method has increased sensitivity, reliability and is less time-consuming than the traditional two-dimensional TLC method.

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Abbreviations: ChoGpl, choline glycerophospholipid; EtnGpl, ethanolamine glycerophospholipid; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; HUVE, human umbilical vein endothelial; PBS, phosphate buffered saline; PlsCho, choline plasmalogen; PlsEtn, ethanolamine plasmalogen; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Ethanolamine glycerophospholipid (EtnGpl) from bovine brain and choline glycerophospholipid (ChoGpl) from bovine heart were purchased from Serdary (London, Canada). Primary cultures of human umbilical vein endothelial cells (HUVE) were used to test the procedure. HUVE cells were collected by treating the luminal surface of the umbilical vein with 0.1% type II collagenase (16). The cells were plated upon human fibronectin-coated glass tissue culture flask. Upon reaching confluency, the cells were replated on 100-mm fibronectin-coated glass tissue culture plates. The endothelial origin was confirmed by the presence of factor VIII antigen (17).

Confluent cells were extracted using *n*-hexane/2-propanol (3:2, vol/vol) (18). Prior to extraction, the medium was removed and the cells were washed with two 3-mL portions of cold phosphate buffered saline (PBS). The plates were then placed immediately on solid CO_2 to minimize damage during extraction (19). Two 3-mL aliquots of *n*-hexane/2-propanol (3:2, vol/vol) were used to extract the cells. Cellular debris was removed by filtration through glass wool. Nitrogen was placed over the extracts to minimize autoxidation of lipids. The extracts were stored at −20°C.

Male Wistar rat (300 g, Harlan, Indianapolis, IN) spinal cords were removed after surgically cutting the dorsal roots followed by hydrostatic expulsion of the spinal cord (20). The spinal cords were immediately frozen in liquid nitrogen. The heads of decapitated rats were frozen in liquid nitrogen and the brains removed on ice in a −4°C cold room. The samples were extracted with *n*-hexane/2-propanol (3:2, vol/vol, 18 mL/g tissue) (21). The lipid extracts were filtered using sintered glass funnels to remove cellular debris.

Prior to phospholipid separation, the lipid extracts were filtered using a Rainin 0.2 μm Nylon filter (Woburn, MA). The sample was dried under nitrogen and redissolved in a known volume of *n*-hexane/2-propanol/water (56.7:37.8:5.5, by vol) prior to HPLC.

The lipid extracts were separated into major phospholipid classes by HPLC. Solvents used were HPLC grade *n*-hexane and 2-propanol from E.M. Science (Cherry Hill, NJ). Solvents were filtered through a 0.5 μm Millipore FH-type Nylon filter (Bedford, MA) and degassed. Solvent A was *n*-hexane/2-propanol (3:2, vol/vol) and solvent B was *n*-hexane/2-propanol/water (56.7:37.8:5.5, by vol). Water was purified using a Millipore water purification system (Bedford, MA). The HPLC instrument consisted of two Altex 100Å pumps (Berkeley, CA), an Altex 420/421 controller and an Altex model 210 injection port. The Dupont (Wilmington, DE) Zorbax Silica column (4.6 mm × 250

mm, 5–6 μm) was maintained at a constant temperature of 34°C with a Jones Chromatography heating block (Littleton, CO). An ISCO (Lincoln, NE) V4 UV variable wave length detector was used to detect peaks at 205 nm.

Two HPLC separations were used for this method. The first separation resolved all of the major phospholipids (22). This permitted the resolution of the EtnGpl and ChoGpl peaks, which were then collected for hydrolysis. The solvent was removed under nitrogen and then the test tubes were inverted over five drops of conc. HCl in a test tube cap for 5 min. This caused the complete hydrolysis of the alkenyl ether bond of the plasmalogens while the alkyl ether, acyl ester and diacyl ester bonds remained intact. The sample was reextracted with *n*-hexane/2-propanol (3:2, vol/vol) and rechromatographed using the second separation system.

The second separation was used to separate the 1-lysophospholipids from the acid-stable fractions of EtnGpl and ChoGpl (Fig. 1). Initial conditions were: column temperature 34°C; flowrate 1.8 mL/min; and solvent composition 55% A/45% B. At 0 min the solvent was changed to 24% A/76% B over a 3-min period. At 8 min the solvent was changed to 100% B over a 3-min period. This separation completely resolved the lysophospholipids from the remaining EtnGpl and ChoGpl. The fractions were collected and quantitated by measuring lipid phosphorus (23). We also used peak area as integrated by a Nelson Analytical 760 series interface (Cupertino, CA) to calculate peak areas and subsequent determination of the plasmalogen proportion with respect to its glycerophospholipid class.

To determine the effect of mild acidic hydrolysis of the alkenyl ether linkage on phospholipid fatty acid composition, ethanolamine glycerophospholipids from astrocytes were subjected to mild acidic hydrolysis for 15 min. The fatty acid compositions of acidic hydrolyzed and nonhydrolyzed EtnGpls were compared.

Phospholipid acyl chains were converted to fatty acid methyl esters (FAME) through base-catalyzed transesterification (24). This method, unlike the method with BF_3 for transesterification, avoids oxidative side reactions and the formation of dimethylacetals from the plasmalogens. FAME were extracted with 2 mL of hexane. The upper phase was pipetted off and the remaining methanol phase washed with two 2-mL aliquots of hexane. FAME were stored in a nitrogen atmosphere at −20°C.

FAME were separated and quantitated by gas-liquid chromatography (GLC). A set of standards (Nu-Chek-Prep, Elysian, MN) were used to establish relative retention times and relative correction factors for each FAME. The internal standard was 17:0 methyl ester. The detector response was linear within the concentration range of the samples for all identified fatty acids of varying chain lengths and degrees of unsaturation with correlation coefficients of 0.985 or greater. This standard was used for the standard curves and for each sample run.

The GLC system was comprised of a Shimadzu (Kyoto, Japan) GC-14A, a Supelco (Bellefonte, PA) SP-2330 capillary column (30-m long) and a flame-ionization detector. Column temperature was maintained at 190°C with nitrogen as the carrier gas at a pressure of 0.5 kg/cm^2. Detector and injector temperatures were maintained at 220°C. A split ratio of 50:1 was used. Peak area data were collected with a Nelson Analytical 760 series intelligent interface and computed with Nelson model 2600 software.

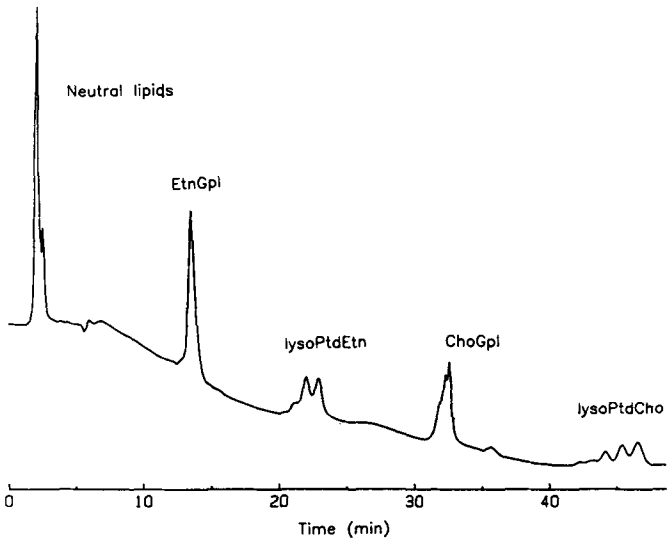

FIG. 1. High-performance liquid chromatographic separation of commercially prepared ethanolamine and choline glycerophospholipid (EtnGpl and ChoGpl) fractions following acidic hydrolysis. The acid-stable ethanolamine and choline glycerophospholipids were separated from the lysophosphatidylethanolamines and cholines (lysoPtdEtn and lysoPtdCho) resulting from the acid labile plasmalogen subclasses. The small peak at ≈35 min represents slight sphingomyelin contamination in the choline glycerophospholipid. The multiple peaks in the fractions are characteristic of the separation and represent partial resolution of molecular species.

RESULTS

Initially we tried to use the method of Renkonen (25) as modified by Yeo and Horrocks (13) with HUVE cell culture samples. In this method the EtnGpl and ChoGpl fractions were collected, solvent removed under a stream of N_2 and dissolved in 1.5 mL $CHCl_3$. Then 1.5 mL of 0.1 M HCl in 95% CH_3OH was added, and the tube was flushed with N_2, capped and vortexed. The sample was incubated at room temperature for 40 min. The ethanolamine plasmalogen (PlsEtn) content ranged from 0 to 100% of EtnGpl for n = 5. The choline plasmalogen (PlsCho) content ranged from 23 to 85% of ChoGpl for n = 3. Such variability suggested this method was unsuitable for samples with EtnGpl or ChoGpl levels at or below 100 nmol.

We used EtnGpl and ChoGpl standards to evaluate the method reported here. The sample sizes were equivalent to cell culture size samples (10–60 nmol) and were subjected to acidic hydrolysis for 1 to 20 min (Fig. 2). PlsCho hydrolysis was complete after 1 min of exposure and PlsEtn hydrolysis was complete after 4 min. No further hydrolysis occurred within the time span of the experiment.

There was little effect of acidic hydrolysis on the fatty acid composition of EtnGpl (Table 1). There was no effect on 20:4n-6 or 22:6n-3 proportions; however, 20:3n-9 proportions were slightly decreased in treated as compared to untreated EtnGpl. Proportions of 18:0 were slightly increased in treated as compared to untreated EtnGpl. These slight alterations, in our experience, would not produce statistically significant alterations.

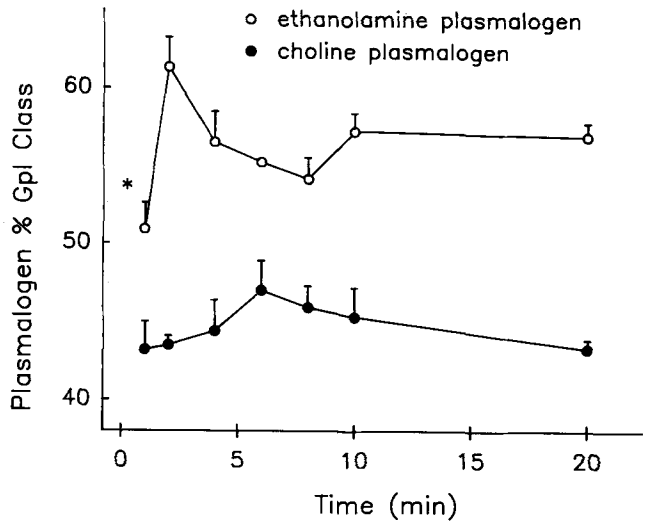

FIG. 2. Concentrated HCl hydrolysis of commercially prepared ethanolamine glycerophospholipid and choline glycerophospholipid. Values are plasmalogen mole % ± SD of the respective glycerophospholipid class and n = 3. For ethanolamine plasmalogen the mole % following 1 min of acidic hydrolysis was less than all other times, $P < 0.05$, Gpl, glycerophospholipid.

TABLE 1

Fatty Acid Composition of Acid-Treated and Untreated Ethanolamine Glycerophospholipid[a]

Fatty acid	Untreated	Treated
16:0	4.1	5.0
16:1	0.6	0.6
18:0	17.5	19.4
18:1	25.0	26.2
18:2	0.3	0.3
20:1	0.4	1.3
20:3n-9	7.1	4.7
20:3n-6	0.4	0.2
20:4n-6	22.0	21.4
20:5n-3	0.8	0.2
22:4n-6	2.4	2.9
22:5n-3	4.7	2.6
22:6n-3	14.7	15.1

[a]Values are fatty acid mole % of ethanolamine glycerophospholipid from non-dibutyryl cyclic adenosine monophosphate treated astrocyte cell cultures in culture for 21 d. Values are n = 1 for each group.

Hydrolysis was quantitated by measuring lipid phosphorus and by peak area integration. Plasmalogen content quantitated by either method produced consistent results (Table 2). The results were not significantly different when comparing between the amount of phospholipid used or by the quantitation method used to determine percent plasmalogen. The plasmalogen contents of HUVE cells, rat spinal cord and rat brain were determined to test the effectiveness of the method on tissue, as well as cell culture size samples (Table 3).

DISCUSSION

A mild acidic hydrolysis procedure for the quantitation of cell culture size samples of plasmalogens is reported here. The procedure is a modification of the Horrocks and

TABLE 2

Comparison of Phosphorus and Peak Area Values for the Quantitation of Plasmalogens[a]

Total nmol	Phosphorus (mole %) ± SD	Phosphorus (mole %) RSD	Peak area (mole %) ± SD	Peak area (mole %) RSD
37.00	82.7 ± 2.5	3.0	76.6 ± 5.9	7.7
18.50	84.7 ± 3.1	3.6	82.9 ± 1.2	1.4
9.25	86.9 ± 4.0	4.6	82.8 ± 3.1	3.7

[a]Values are expressed as mole % ± SD of glycerophospholipid class of semi-purified ethanolamine plasmalogen. Total nmoles represents the total number of nmoles used for the acidic hydrolysis. For each value (n = 3) with analysis done in triplicate. The relative standard deviation (RSD) represents both inter- and intra-sample variability.

TABLE 3

Mole % of Plasmalogen in EtnGpl and ChoGpl[a]

	% EtnGpl	% ChoGpl
Rat spinal cord	73.56 ± 1.43	5.7 ± 0.2
Rat brain	55.94 ± 2.64	12.5 ± 1.9
HUVE cells[b]	40.5 ± 1.90	11.6 ± 0.9

[a]Values are mole % of glycerophospholipid class lipid phosphorus. For rat spinal cord and brain values, n = 3. For HUVE cell values are for n = 4. EtnGpl, ethanolamine glycerophospholipid; ChoGpl, choline glycerophospholipid; HUVE, human umbilical vein edothelial.

[b]Refer to Reference 31.

Sun procedure (15) and utilizes the acid lability of the alkenyl ether linkage in the *sn*-1 position. HPLC was used to separate the phospholipids. Using HPLC to separate phospholipids permits good recovery of all phospholipids, improved phosphorus determination and detection of smaller changes in phospholipid composition with statistical significance due to a lower variance than with TLC (26). The method of Rotstein *et al.* (10) involves the use of a two-phase system to extract the lipids following hydrolysis. Using *n*-hexane/2-propanol (3:2, vol/vol) for all extraction procedures avoids the need for a two-phase system with the losses of lysoglycerophospholipids into the water phase (27). Thus, this procedure optimizes conditions for the separation and quantitation of cell culture size samples of EtnGpl and ChoGpl for plasmalogen content.

Initially, we used the method of Yeo and Horrocks (13) for the hydrolysis of the alkenyl ether. This procedure appeared to completely hydrolyze the fatty acids from the phospholipid, presumably because the samples sizes were very small. The method reported here caused the complete hydrolysis of the plasmalogen linkage while the alkylacyl and diacyl fractions remained intact. After 1 and 4 min, hydrolysis was completed for ChoGpl and EtnGpl, respectively. No more hydrolysis occurred between 4 and 20 min of exposure to acid fumes. Acidic hydrolysis of EtnGpl fraction from astrocytes for 15 min did not significantly alter the phospholipid fatty acid composition.

Literature values were obtained using EtnGpl from bovine brain (28) and ChoGpl from bovine heart (29) over this time period. We used this method to determine the plasmalogen content of EtnGpl and ChoGpl in HUVE cells, rat spinal cord and rat brain. Blank *et al.* (30) found the same plasmalogen content in HUVE cells as we did

with 42.5% PlsEtn in EtnGpl and 6.8% PlsCho in ChoGpl. Using our method, we obtained literature values for plasmalogens from several different sources and species without altering the fatty acid composition. The rapidity of the method, analysis time of approximately 2-3 h depending upon the resolution needed in the first separation, is superior to two-dimensional TLC methods. Furthermore, there are no effects of humidity on the day-to-day separation as seen in TLC. This simple method is suitable for use with cell cultures and for following plasmalogen metabolism.

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COMMUNICATION

Time Response of Cholesterol Synthesis Inhibition by Compactin-Related Compounds. *In vitro* Quantitation of the "Escape Phenomenon"

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The time course of the inhibition of cholesterol synthesis by low and high doses of mevinolin and monacolin X were studied in normal human skin fibroblasts, fibroblasts without low density lipoprotein receptor and HepG2 hepatoma cells. Low doses of the inhibitors (0.2 ng/mL) caused a sharp decrease in the rate of cholesterol synthesis during the first 2–3 h, which gradually increased to about 40% during the next 6 h. Further incubation led to a decrease or stabilization of the cholesterol synthesis rate. High doses of the drugs (100 mg/mL) strongly inhibited cholesterol synthesis during the first 2–3 h, followed by a moderate increase during the next 20 h. No drug or tissue selectivity was observed.

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Compactin-related compounds are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol synthesis (1). Associated with the inhibition of cholesterol synthesis by these compounds is an interesting response called "escape phenomenon" (2,3) which is characterized by a many-fold increase in HMG-CoA reductase and HMG-CoA synthase activity in cultured cells. The phenomenon is mainly due to the activation of HMG-CoA reductase and HMG-CoA synthase genes in response to the lowering of the regulatory sterol concentration (4). Although the molecular mechanisms of this phenomenon are being studied intensively, only limited information is available about the time course, the dose dependence and the possible tissue selectivity associated with the drug's action. These parameters may provide important clues as to the events associated with the "escape phenomenon."

MATERIALS AND METHODS

Cells. Human skin fibroblasts from healthy donors and from donors affected by homozygous familial hypercholesterolemia (FH) (the FH fibroblasts were a kind gift from Dr. I.V. Fuki), and human hepatoma cells HepG2 were maintained as previously described (5). Cells were grown to confluency in 24-well multidishes (Costar, Cambridge, MA); average protein concentration was about 100 μg per well.

Cholesterol synthesis. At the beginning of the experiment the medium was changed and replaced with medium (final volume 0.5 mL) containing a low (final concentration 0.2 ng/mL) or high (final concentration 100 ng/mL) dose of mevinolin or monacolin X (6). The rate of cholesterol synthesis was determined by measuring incorporation of sodium [2-^{14}C]acetate (Amersham, Bucks, England; specific activity 40–60 mCi/mmol; final concentration 6 μCi/mL, 0.12 nM) into digitonin precipitable sterols during a 2-h incubation as previously described (5). Incubation time is defined as the total time of incubation in the presence of the drug (*i.e.*, time of incubation with the drug alone plus 2 h incubation with the drug and [^{14}C]acetate). The zero time point refers to the cholesterol synthesis rate in the absence of the drugs. Statistical significance was calculated using Student's *t*-test.

RESULTS

The time course of inhibition of cholesterol synthesis by mevinolin in healthy human skin fibroblasts is shown in Figure 1. The low dose of mevinolin (0.2 ng/mL) after 2 h incubation caused an inhibition of cholesterol synthesis by about 25% ($P < 0.01$). Longer incubation in the presence of mevinolin caused a pronounced increase in cholesterol synthesis rate. After 3 h, the rate had returned to control levels. Comparison of the cholesterol synthesis rate after 2 and 8 h of incubation with a low dose of mevinolin gave a 39% stimulation of cholesterol synthesis during this time period. A high dose of mevinolin (100 ng/mL) caused a 76% inhibition of cholesterol synthesis after 2 h of incubation ($P < 0.001$). Further incubation of the cells with mevinolin at this dose did not cause statistically significant changes in cholesterol synthesis rate.

Exposure to a low dose of mevinolin for 3 h inhibited cholesterol synthesis in human skin fibroblasts lacking low density lipoprotein (LDL) receptors by 36% ($P < 0.01$) (Fig. 2). Longer incubation led to a return of the cholesterol synthesis rate to the control level by the eighth hour; however, by the twentieth hour the rate decreased again (37% inhibition; $P < 0.01$ *vs.* control). Incubation with a high dose of mevinolin for 3 h inhibited cholesterol synthesis by 86% ($P < 0.001$), which was followed by a gradual but statistically significant increase of this rate ($P < 0.001$ 2 h *vs.* 24th h). It should be noted that the absolute rate of cholesterol synthesis in FH fibroblasts was fivefold higher than in healthy cells.

The response to mevinolin by HepG2 cells (Fig. 3A) was quantitatively similar to that in skin fibroblasts without LDL receptors. Low doses of monacolin X, another less potent compactin-related compound, caused 10% inhibition ($P < 0.05$) of cholesterol synthesis during the initial 2–3 h, followed by an increase in the cholesterol synthesis rate up to 123% of the control after 8 h of incubation ($P < 0.01$) (Fig. 3B). A total increase by 37% was observed from the second to the eighth hour of incubation.

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Abbreviations: FH, familial hypercholesterolemia; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoproteins.

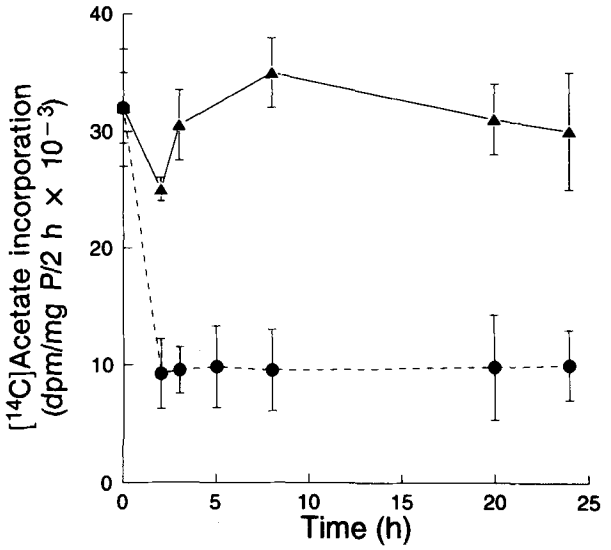

FIG. 1. Time response of cholesterol synthesis inhibition by mevinolin in healthy human skin fibroblasts. Cells were incubated at 37°C for the indicated periods of time in the absence or presence of mevinolin added at a final concentration 0.2 ng/mL (▲) or 100 ng/mL (●). Two hours before the end of the incubation, sodium [^{14}C]acetate was added to the incubation mixture at a final concentration of 6 μCi/mL. After incubation, cells were washed and incorporation of [^{14}C]acetate into digitonin precipitable sterols was determined. Each point represents the mean ± SE of the mean of triplicate or quadruplicate determinations.

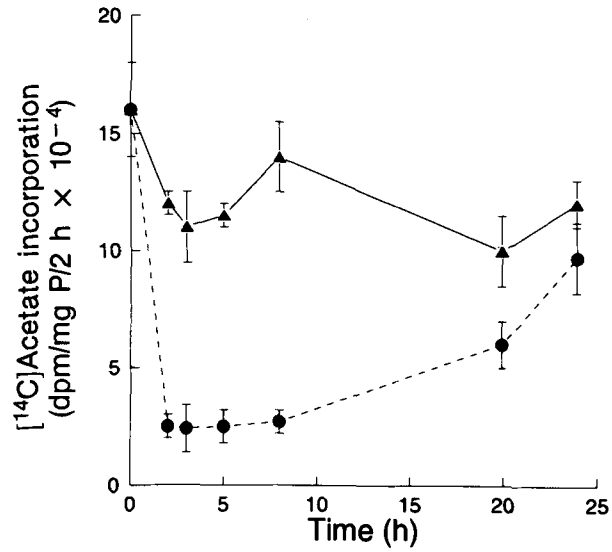

FIG. 2. Time response of cholesterol synthesis inhibition by mevinolin in skin fibroblasts from a patient affected by homozygous familial hypercholesterolemia. Cells were incubated at 37°C for the indicated periods of time in the absence or presence of mevinolin added at a final concentration 0.2 ng/mL (▲) or 100 mg/mL (●). Two hours before the end of the incubation, sodium [^{14}C]acetate was added to the incubation mixture at a final concentration 6 μCi/mL. After incubation, cells were washed and incorporation of [^{14}C]acetate into digitonin precipitable sterols was determined. Each point represents the mean ± SE of the mean of triplicate or quadruplicate determinations.

Further incubation of the cells with monacolin X resulted in a gradual decrease of the cholesterol synthesis rate back to the control level. A high dose of monacolin X inhibited cholesterol synthesis by 53% after a 2-h incubation

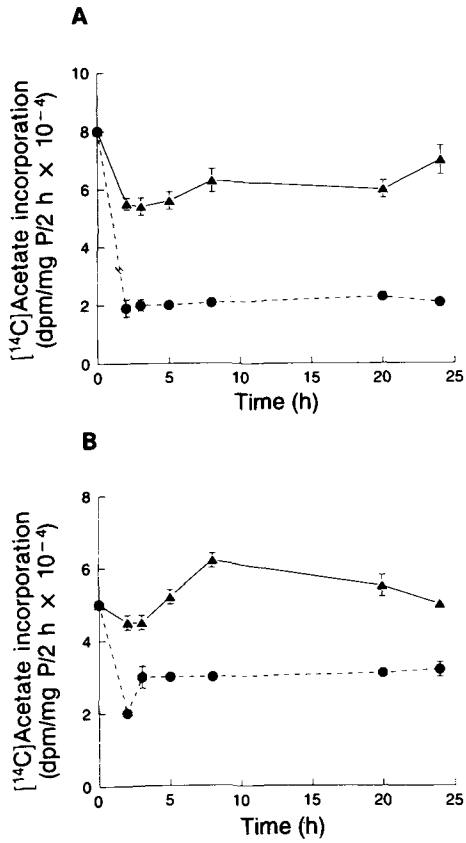

FIG. 3. Time response of cholesterol synthesis inhibition by mevinolin (A) and monacolin X (B) in HepG2 cells. Cells were incubated at 37°C for the indicated periods of time in the absence or presence of mevinolin (A) or monacolin X (B) added at a final concentration 0.2 ng/mL (▲) or 100 ng/mL (●). Two hours before the end of the incubation, sodium [^{14}C]acetate was added to the incubation mixture at a final concentration 6 μCi/mL. After incubation, cells were washed and incorporation of [^{14}C]acetate into digitonin precipitable sterols was determined. Each point represents the mean ± SE of the mean of triplicate or quadruplicate determinations.

($P < 0.01$) followed by a subsequent increase of the cholesterol synthesis rate (38% inhibition after 24 h incubation). A similar response of the other two cell types on the inhibition of cholesterol synthesis by monacolin X was observed (data not shown).

DISCUSSION

In this study, we compared the inhibition of cholesterol synthesis in three human cell types that had been treated for various time periods with two compactin-related compounds at different doses. The low dose (0.2 ng/mL) corresponded to the nonsaturating active dose of compactin-related compounds on the cell types chosen (5). The high dose (100 ng/mL) corresponded to the concentration of Mevacor (trade name of mevinolin) in the plasma of patients treated with this drug (60–100 mg/d) (7,8). Mevinolin is the stronger cholesterol synthesis inhibitor, while monacolin X is a less potent inhibitor. The cells were from healthy humans and FH patients, *viz.* healthy and LDL-receptor defective fibroblasts; HepG2 cells were used as a model derived from the major cholesterol producing organ. Drugs were present in the incubation mixture while

the cholesterol synthesis rate was measured, thus modeling the situation of continuous administration of the drug.

In the experiments with a low dose of the drug, cholesterol synthesis inhibition reached a maximum in all cell types after 2–3 h of incubation. Further incubation, however, resulted in an increase of the cholesterol synthesis rate. This allows the assumption that it takes 2–3 h for the drug to penetrate into the cell, to activate feedback mechanism(s) and to stimulate the rebound of HMG-CoA reductase and HMG-CoA synthase. This is consistent with the rebound activation time found by Stone *et al.* (3) in mononuclear leukocytes of patients treated with lovastatin. The growth of cholesterol synthesis rate continued up to the eighth hour of incubation, similar to what was observed by Hagemenas and Illingworth (8) and Stone *et al.* (3). Very similar increases in cholesterol synthesis rates were noted between the third and eight hour of incubation in all experiments, *i.e.*, 25–39%. The rates did not depend on either the level of inhibition during the first 2–3 h of incubation nor on the basal rate of cholesterol synthesis. While in absolute terms, the effect of mevinolin on cholesterol synthesis in FH fibroblasts was much more pronounced than in healthy cells, the relative effects were very similar. It should be noted, however, that while incubation with the drugs should provide a steady supply of the drugs to the cells, it cannot be excluded that the drugs metabolize, which would change their effective concentration.

Analysis of the time response of inhibition of cholesterol synthesis by the high drug dose showed a rapid inhibition of cholesterol synthesis after 2–3 h of incubation followed by a gradual increase in the cholesterol synthesis rate. This is consistent with a rebound of HMG-CoA reductase in an excess of free inhibitor. The only difference was observed in LDL-receptor defective fibroblasts where the increase in cholesterol synthesis rate was more rapid than in the other cells. The cause of this effect could be associated with the relatively higher absolute rate of cholesterol synthesis in these cells as demonstrated in this study and earlier by Shireman *et al.* (9). The results are also consistent with the notion that continuous administration of a high dose of the drug may be less effective in FH patients than low doses (7,8). No tissue or drug selectivity was observed.

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Oxidative Modification of Triglyceride-Rich Lipoproteins in Hypertriglyceridemic Rats Following Magnesium Deficiency

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Hypertriglyceridemia observed in magnesium (Mg)-deficient rats was associated with a significant increase in the very low-density lipoprotein (VLDL) plus low-density lipoprotein (LDL) fractions. The results from *in vitro* copper-induced lipid peroxidation, expressed in terms of conjugated dienes and thiobarbituric acid reactive substances content, showed that VLDL + LDL particles from Mg-deficient rats were more susceptible to oxidative damage than lipoproteins from control rats. These results suggest that the mechanism responsible for the atherogenicity and tissue damage characteristic of Mg deficiency may be mediated by an increased susceptibility of triglyceride-rich lipoproteins to peroxidation in hypertriglyceridemic animals.
Lipids 28, 573-575 (1993).

Several studies have provided evidence that magnesium (Mg) deficiency affects lipid metabolism (1,2) and is associated with tissue injury affecting the physical state of bilayer membrane lipids (3). Mg deficiency has been implicated as a risk factor in the development of atherosclerosis. Early studies indicate that Mg deficiency enhances vascular lipid infiltration in rats, rabbits and monkeys fed atherogenic diets (4). Recent studies have confirmed that Mg deficiency can intensify cardiovascular lipid deposition and formation of lesions in animals on atherogenic diets and that dietary Mg supplementation can prevent atherosclerosis (5). Mg deficiency affects lipoprotein metabolism (4,6); however, there has not been agreement that formation of cardiovascular lesions is necessarily correlated with cholesterolemia in Mg-deficient animals (5). The mechanism causing vascular lesions has yet to be identified. An important characteristic of hyperlipemia associated with Mg deficiency in rats is the post prandial accumulation of triglyceride-rich lipoproteins (TGRLP) (7). The relationship between hypertriglyceridemia, TGRLP and atherosclerosis remains obscure, but data suggest that the mechanism responsible for the atherogenicity of the hypertriglyceridemic state may be related to the increased susceptibility of TGRLP to peroxidation (8). The apoprotein B (apoB) molecules contained in TGRLP of hypertriglyceridemic subjects may become partially degraded and such alterations could promote binding to macrophages which act as scavenger cells. The uptake of oxidized lipoproteins by macrophages would lead to accumulation of esterified cholesterol and formation of foam cells (9). Several previous studies have shown that Mg deficiency increased lipid peroxidation (2,10,11). Mg-deficient animals had lower levels of vitamin E in plasma and tissues, and the protection by vitamin E of myocardial necrosis induced by Mg deficiency, as shown recently would suggest that free radicals participate in Mg deprivation-induced injury (12). The present investigation was undertaken to determine the effect of Mg deficiency in rats on the susceptibility of TGRLP to peroxidation.

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Abbreviations: AAS, atomic absorption spectrophotometry; apo, apoprotein; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Mg, magnesium; TBARS, thiobarbituric acid reactive substances; TGRLP, triglyceride-rich lipoproteins; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Thirty-six weanling male Wistar rats (Iffa-Credo, L'arbresle, France) (≈60 g; 3-wks-old) were divided randomly into Mg-deficient and control groups (18 animals per group). The institution's guide for the care and use of laboratory animals was followed. The animals were pair-fed with the appropriate diets for 8 d using an automatic feeding apparatus. Distilled water was provided *ad libitum.* Diets contained (g/kg) 200 casein, 3 DL-methionine, 702 sucrose, 50 corn oil, 35 mineral mixture and 10 vitamin mixture as described previously (13). Mg levels determined by flame atomic absorption spectrophotometric analysis (model 400, Perkin Elmer, Norwalk, CT) were 30 (deficient) and 990 mg/kg (control). Experiments were performed in nonfasting rats. Plasma from 18 animals per group were obtained from rats anesthetized with sodium pentobarbital (40 mg/kg of body weight). Equal volumes of plasma samples from three animals were pooled for blood analysis and lipoprotein separations.

Ultracentrifugation was performed (14) at 15°C in a Beckman L5-50 model ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) with a Ti-50 titanium rotor. Ethylenediaminetetraacetic acid (EDTA) (1 mg/mL), and butylated hydroxytoluene (BHT) (4.4 μg/mL) were added immediately before lipoprotein separation. Samples were overlayered with 0.15 M NaCl (d = 1.006 g/mL), and chylomicrons were discarded following two centrifugations for 30 min at 12,000 × *g*. In order to isolate the VLDL + LDL (very low density lipoproteins and low-density lipoprotein) fraction, the infranatant was adjusted to density 1.050 g/mL with solid KBr. The centrifugation was done at 100,000 × *g* for 20 h. The VLDL + LDL fractions were then washed by a further period of ultracentrifugation at the same density. Before oxidation experiments, the purified lipoprotein fractions were dialyzed against 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, which was made oxygen-free by vacuum degassing followed by purging with nitrogen.

Oxidation experiments were performed as described by Esterbauer *et al.* (15). The EDTA/BHT-free dialyzed lipoproteins were diluted with dialysis buffer to a final concentration of 0.2 mg protein/mL. The same concentrations were used for both experimental groups. Oxidation was initiated by addition of freshly prepared $CuSO_4$ solution at various concentrations (5, 25 and 50 μM). The kinetics of the oxidation of lipoproteins were determined by monitoring the change in the 234 nm absorbance at 37°C on a spectrophotometer Uvikon 820 (Kontron, St-Quentin-en-Yvelines, France) using appropriate dilutions. The in-

itial absorbance at 234 nm was set to zero and the increase in absorbance was recorded. Thiobarbituric acid reactive substances (TBARS) were determined in lipoproteins (16) after lipid peroxidation with 50 μM $CuSO_4$. Plasma Mg was determined with a Perkin-Elmer model 400 AAS after dilution in lanthanum chloride solution containing 1 g/L La. Triglycerides (Biotrol, Paris, France), cholesterol and phospholipids (Biomérieux, Charbonnières-les-Bains, France) were determined in plasma by enzymatic procedures (17–19). Protein concentration of isolated lipoproteins was determined by a modified Lowry method using bovine serum albumin, fraction V (Sigma, L'Isle d'Abeau, France) as a standard (20).

Results are expressed as means ± SEM. Statistical significance of differences between means was assessed by Student's *t*-test.

RESULTS AND DISCUSSION

Mean final body weights of Mg-deficient and pair-fed control rats were 87 ± 3 and 96 ± 2 g (mean ± SEM; n = 18; $P < 0.01$), respectively. Hypomagnesemia was usually observed in Mg-deficient rats (0.13 ± 0.01 *vs.* 0.83 ± 0.03 mM in control rats, $P < 0.001$). Triglyceride plasma levels were significantly higher in Mg-deficient rats than in controls, whereas plasma cholesterol was not altered (Table 1). Hypertriglyceridemia observed in Mg-deficient animals was associated with a significant increase in the VLDL + LDL fraction levels (Table 1). VLDL + LDL fractions from Mg-deficient rats as compared to control rats exhibited increased oxidation *in vitro* as monitored by conjugated diene formation (Fig. 1) and by TBARS activity (Fig. 2).

Previous studies from our laboratory have provided evidence that experimental Mg deficiency in rats produced a dyslipoproteinemia characterized by increased plasma levels of VLDL and LDL and decreased plasma high-density lipoprotein (HDL) (13). The mechanisms behind the hyperlipemia of Mg-deficient rats have been addressed in several studies (6). A recent study indicated that decreased clearance of circulating triglycerides is a major mechanism contributing to hyperlipemia in Mg-deficient rats (7). The hypertriglyceridemic state in humans may act indirectly on the processes leading to atherosclerosis by influencing the metabolism and composition of intermediate density lipoproteins, LDL and HDL (8). There are data that suggest that delayed removal of TGRLP in the hypertriglyceridemic state might contribute to development of cardiovascular disease by uptake of these particles by macrophages (21).

Our results demonstrate that Mg deficiency affects the susceptibility of TGRLP to *in vitro* peroxidation. Because both LDL and VLDL undergo lipid peroxidation and rats have a relatively small amount of LDL, we chose to investigate the effect of Mg deficiency in rats on *in vitro*

TABLE 1

Effect of Dietary Magnesium (Mg) on Concentrations of Plasma Lipids and Lipoproteins[a]

	Control (mg/100 mL)	Mg-deficient (mg/100 mL)
Plasma[a]		
Triglycerides	76 ± 8	290 ± 46[c]
Total cholesterol	44 ± 4	47 ± 8
VLDL + LDL[a]		
Triglycerides	54 ± 6	179 ± 20[c]
Total cholesterol	14 ± 1	30 ± 3[c]
Phospholipids	20 ± 2	56 ± 6[c]
Proteins	15 ± 2	30 ± 4[b]

[a]Means of six determinations per group ± SEM, VLDL, very low density lipoproteins; LDL, low-density lipoproteins.
[b]Significant difference from control rats, $P < 0.01$.
[c]Significant difference from control rats, $P < 0.001$.

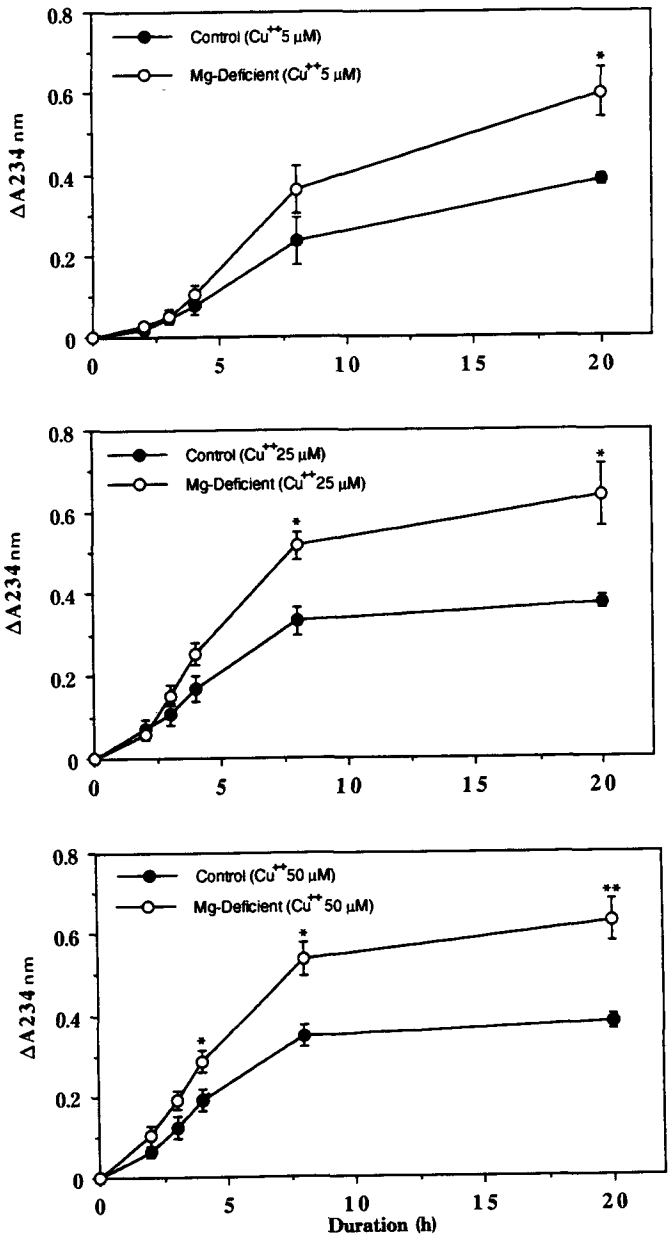

FIG. 1. Rate of formation of conjugated dienes during Cu^{++} stimulated oxidation of very low density lipoprotein + low-density lipoprotein fractions isolated from control and magnesium (Mg)-deficient rats. The results represent the mean (± SEM) of six determinations per group. Those points that were significantly different between control and Mg-deficient rats are designated with an asterisk. *$P < 0.01$; **$P < 0.001$).

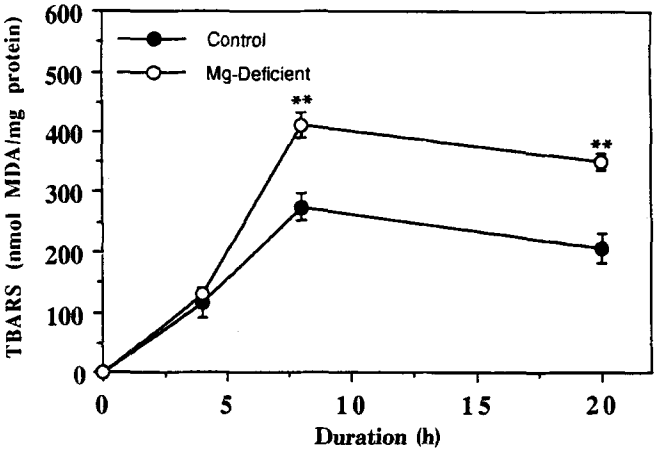

FIG. 2. Production of thiobarbituric acid reactive substances (TBARS) during Cu^{++} (50 μM) stimulated oxidation of very low density lipoproteins + low-density lipoprotein fractions isolated from control and magnesium (Mg)-deficient rats. The results represent the mean (± SEM) of six determinations per group. Those points that were significantly different between control and Mg-deficient rats are designated with an asterisk. $^{**}P < 0.001$. MDA, malondialdehyde.

oxidation of the lipoprotein fraction containing both LDL and VLDL (22). Plasma lipid peroxides have also been reported to be elevated in diabetic rats when plasma triglycerides are increased, with TBARS levels being elevated in lipoprotein fractions containing both VLDL and LDL (23). Enhanced production of lipid peroxides may be attributable either to changes in the lipid composition or to a depleted lipoprotein antioxidant system (24). In lipoproteins from Mg-deficient animals, there is an increase in triglyceride/protein and phospholipid/protein ratios (25) which could theoretically explain the higher rate of oxidation of lipoproteins. Other studies have shown changes in fatty acid metabolism (2) and in fatty acid composition of lipoproteins in Mg-deficient rats as compared to controls, *i.e.*, an increased percent composition of linoleic acid (25). As no information is available concerning the influence of Mg on antioxidant concentration in lipoproteins, further studies will be required to pinpoint the mechanism by which lipoprotein oxidation increases in Mg-deficient animals.

Several studies indicate that TGRLP, when oxidized, become toxic to cells in culture and that oxidatively modified apoB-containing lipoproteins play a role in the recruitment and retention of macrophages. Macrophages themselves are responsible for oxidation and resultant toxicity because of their highly developed oxidation capacity (21,26). Recent studies have demonstrated that such macrophages are more abundant in animals fed diets low in magnesium (5). Thus, increased oxidation of TGRLP combined with the increased abundance of macrophages in Mg-deficient animals may result in excessive uptake of the modified lipoproteins by the scavenger receptor, converting macrophages into foam cells. The results of this study suggest that the mechanism responsible for the atherogenicity and tissue damage characteristic of Mg deficiency may be mediated by increased susceptibility of TGRLP to peroxidation in hypertriglyceridemic animals.

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ARTICLE

Inositol 1,4,5-Trisphosphate Accumulation in Brain of Lithium-Treated Rats

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The mechanism of action of lithium as a drug for patients with affective disorders was investigated. Three-week-old male rats were orally administered 2.7 mEq Li_2CO_3/kg/d for 1 or 3 wk, and phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol phosphate (IP), inositol diphosphate (IP_2) and inositol triphosphate (IP_3) levels in brain were measured. The levels of IP were increased 1.7 and 2.4 times after 1 wk and 3 wk of lithium administration, respectively, while PI, PIP, PIP_2, IP_2 and IP_3 levels were not altered. IP_3 was further fractionated by high-performance liquid chromatography into I-1,3,4-P_3 and I-1,4,5-P_3. In the control rat brain, the relative percentages of I-1,3,4-P_3 and I-1,4,5-P_3 were 95.8 and 4.2, respectively. However, after 3 wk of lithium administration, the values were changed to 69.6 and 30.3%, respectively. This increase in the neurotransducer I-1,4,5-P_3 in the brain may be relevant to the mechanism of action in the lithium treatment of patients with manic-depressive disorders. *Lipids 28,* **577-581 (1993).**

Lithium is used for the treatment of manic-depressive illness and other psychiatric disorders (1). Although the mechanisms of the therapeutic action of lithium is not known, this treatment has various endocrine and metabolic effects on the brain (2). The effects of lithium on the metabolism of inositol phosphates (IPs) and inositol phospholipids (3) are among the possible mechanisms involved in the pharmacological action of lithium, since breakdown of inositol phospholipids plays a role in the transducing mechanism of receptor occupation by several neurotransmitters (4). Sherman *et al.* (5) discovered that lithium is a strong inhibitor of inositol phosphate 1-phosphatase. Through this action, lithium administration causes the accumulation of inositol monophosphate and a decrease in the myoinositol level in rat brain (6). These findings suggest that lithium may inhibit the resynthesis of inositol phospholipids resulting in gradual depletion of the inositol phospholipids of the membrane. This might also gradually decrease the levels of IPs, which play an important role in signal transducing mechanisms (7). Clinical observations indicate that at least 2 to 3 wk of lithium treatment is required to achieve an effect in patients with affective disorders (1).

The effects of chronic lithium administration on the levels of several phospholipids and inositol 1-phosphate (I-1-P), as well as an I-1-P phosphatase activity, in rat brain have previously been investigated (5). On the other hand, inositol triphosphate (IP_3) levels have not yet been reported. Inositol-1,4,5-trisphosphate (I-1,4,5-P_3) is known as a key transducer of input signals to output transmitters (8). We therefore estimated the levels of inositol phospholipids, *i.e.*, phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) and of IPs, *i.e.* IP, inositol diphosphates (IP_2) and IP_3 in rat brain after chronic lithium administration. In particular, we examined the change in the ratio of I-1,4,5-P_3 to I-1,3,4-P_3 in IP_3. A preliminary report has been published (9).

MATERIALS AND METHODS

Chemicals. Myo-[^{3}H]inositol (2.96 TBq/mmol), D-myo-[2-^{3}H]I-1-P (37 GBq/mmol), D-myo-[2-^{3}H]inositol 1,4-bisphosphate (37 GBq/mmol), and D-[2-^{3}H]inositol 1,3,4,5-tetrakisphosphate (37 GBq/mmol) were purchased from Amersham (Buckinghamshire, United Kingdom). I-1,3,4-P_3 (370 GBq/mmol), [2-^{3}H-*inositol*]PIP (74-370 GBq/mmol), and [2-^{3}H-*inositol*]PIP_2 (74-370 GBq/mmol) were purchased from Dupont/New England Nuclear (Boston, MA). Precoated silica gel thin-layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). All other chemicals were of reagent grade; solvents were distilled before use.

Chronic lithium administration. Three-week-old male Sprague-Dawley rats (eight rats per group) were orally administered with 2.7 mEq Li_2CO_{33}/kg/d for one week or three weeks. At 24 h after the last administration, the rats were decapitated, and the brains were removed as soon as possible (within 15 s) and cooled in an ice-cold isotonic saline solution. After being blotted dry, the brains (1.5 to 2.0 g) were weighed and homogenized in 4 mL of 5% (wt/vol) trichloroacetic acid for measurement of IPs or 4 mL of 0.25 M sucrose for measurement of PI phosphates. A control group of eight rats was fed the same diet without lithium.

Inositol polyphosphate analysis. The homogenized brains in 5% trichloroacetic acid were centrifuged for 5 min at 1000 × *g*. Aliquots (1 mL) of supernatant solution were supplemented with 185 Bq of [^{3}H]IP, [^{3}H]IP_2 and [^{3}H]IP_3. [^{3}H]Inositol tetraphosphates (IP_4) were also added for high-performance liquid chromatography (HPLC). Then the samples were extracted three times with 5 mL each of diethyl ether to remove the trichloroacetic acid.

Separation of inositol polyphosphates by ion exchange resin column chromatography. The samples were diluted with 5 mL of water for application to a Dowex-1-8X ion exchange column (Dow Chemical Co., Midland, MI) as

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Abbreviations: DPO, 2,5-diphenyloxazole; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; I-1,3,4-P_3, inositol 1,3,4-trisphosphate; I-1,4,5-P_3, inositol 1,4,5-trisphosphate; IP, inositol phosphate; I-1-P, inositol 1-phosphate; IP_2, inositol diphosphate(s); IP_3, inositol triphosphate(s); IP_4, inositol tetraphosphate(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; POPOP, 1,4-*bis*[2-(5-phenyloxazolyl)]benzene; TLC, thin-layer chromatography.

described by Downes and Michell (10). The column was eluted with 15 mL of water, 15 mL of 0.2 M ammonium formate/0.1 M formic acid (IP), 15 mL of 0.4 M ammonium formate/0.1 M formic acid (IP_2) and 15 mL of 1 M ammonium formate/0.1 M formic acid (IP_3,IP_4). Aliquots were dissolved in scintillation fluid [Triton X-100/toluene (1:2, vol/vol) containing 8.6 g of 2,5-diphenyloxazole (DPO) and 0.26 g of 1,4-*bis*[2-(5-phenyloxazolyl)]benzene (POPOP)/2.4 L of Triton/toluene] and radioactivity was counted. The remainder was lyophilized and fractionated by HPLC.

Separation of inositol polyphosphates by HPLC. The lyophilized samples containing IP were diluted to 1 mL, and one-third was injected into the HPLC. A precolumn of silicic acid (4 × 250 mm; mounted before the injector loop), a Partisil SAX guard column (Whatman, Clifton, NJ; 4.6 × 50 mm) and a Partisil SAX HPLC separating column (4.6 × 250 mm; Cobert Associates, St. Louis, MO) were used as described by Wilson *et al.* (11). The column was eluted with a gradient system of (A) 50 mM ammonium formate, pH 3.8 and (B) 2.7 M ammonium formate, pH 3.8, as follows: 0 min, 0% B; 10 min, 0% B; 30 min, 70% B; 50 min, 100% B; 70 min, 100% B; 70.01 min, 0% B; flow rate 1 mL/min; fractions of 1 mL were collected. Aliquots (0.5 mL) of each fraction were removed and to each aliquot was added 1 mL of water and 10 mL of scintillation fluid for radioactivity counting. Xylitol (20 nmol) was added to the remainder of the samples to check recovery; then the samples were lyophilized.

Digestion of inositol polyphosphates. After lyophilization, the dried residues were digested by alkaline phosphatase (from bovine intestinal mucosa, Type 7-N, Sigma, St. Louis, MO) as described by Rittenhouse and Sasson (12). The incubation conditions were 20 mM ethanolamine, 5 mM $MgCl_2$, 76 unit/mL of enzyme, pH 9.6, total volume μL/tube at 37°C for 5 h. The samples were then desalted with Amberlite MB-2 (mixed ion exchange resin), and inositol was eluted with water.

Derivatization and gas chromatography. The resulting inositol samples were dried under vacuum and 10 nmol of mannitol was added as an internal standard. Then half the sample was removed for radioactivity counting to check the recovery. The remainder was dried under N_2. At this step, the recovery of [^{3}H]inositol was 40–60%. Then 100 μL of a mixture of dry pyridine, trimethylchlorosilane and hexamethyldisilazane (5:2:1, by vol) was added to each sample. The samples were capped and heated for 30 min at 55–60°C. Then 3 mL of water and 3 mL of chloroform were added to each sample to extract the pyridine with water. The water phase was removed, and the chloroform phase was dried under an N_2 flow. The residue was taken up in 2–3 μL of hexane and 1 μL was injected at 260°C onto a 25-m capillary fused silica CBP-5 column (Shimadzu Co., Tokyo, Japan) using He as carrier gas (column temperature, 210°C). Inositol, xylitol and mannitol were quantitated by integration of the flame-ionization detector peak areas. Final results were calculated by considering separately the recoveries of xylitol, mannitol and [^{3}H]inositol.

PI extraction. Total lipids were extracted from the homogenate (0.8 mL), including 185 μBq of [^{3}H]PI, with chloroform/methanol (1:2, vol/vol). The mixture was stirred for 20 min, then 1 mL of chloroform and 1 mL of water were added to each sample. The lower phase was collected, and the upper phase was reextracted twice with 1 mL of chloroform as described by Bligh and Dyer (13).

PI separation. The chloroform layer was evaporated, and the lipids were analyzed by two-dimensional TLC as described by Rouser *et al.* (14). The lipid fractions were detected under ultraviolet light after spraying the plate with 0.001% primuline in acetone/water (4:1, vol/vol). The PI fractions were scraped off the plate and placed into vials for radioactivity counting or into tubes to measure phosphorus content (14).

PIP and PIP_2 extraction. The homogenates including 185 μBq of [^{3}H]PIP and [^{3}H]PIP_2 were washed twice with 7.5 mL of chloroform/methanol (1:1, vol/vol), (containing 60 μmol/g tissue of $CaCl_2$) to remove other major phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), *etc.*]. Then 5 mL of chloroform/methanol/conc. HCl (200:100:1, by vol) was added, and the samples were stirred for 1 h. One mL of 1 N HCl was added, and the mixture was stirred and centrifuged. The lower phase was collected and the upper phase and interface were reextracted twice with 3.5 mL of fresh lower phase.

PIP and PIP_2 separation. The extract was dried under a stream of nitrogen and applied to high-performance thin-layer chromatography (HPTLC) plates which had been treated with oxalate and heated at 110°C for 30 min as described by Schacht (15). The developing solvent was chloroform/methanol/ammonia/water (90:70:5:15, by vol). The adsorbent bands were scraped off, and the phosphorus contents or radioactivities were estimated as described above.

Lithium analysis. Lithium concentration in blood sera and tissues was determined with a modified atomic absorption spectrophotometric method developed for determining lithium in blood serum (16). The atomic absorption spectrophotometer used was a Model 703 (Perkin-Elmer Corp., Norwalk, CT) with either a single slot burner or a three-slot burner. The lithium hollow-cathode lamp was obtained from Perkin-Elmer Corp. It was used at a wavelength of 668 nm, a slit width of 2, a scale expansion of 1 and a source current of 15 mA. The gas pressure was 8.5 lbs/in for N_2, and the flow rate was 9 mL/s. Standards were analyzed by adding lithium to control tissues and following the same procedure.

Student's t-test. All experiments were repeated several times (n = 3–8) and analyzed by Student's *t*-test.

RESULTS

The lithium content of rat brain after oral administration (2.7 mEq/kg/d) of lithium carbonate is shown in Table 1. The results are in excellent agreement with those of Sherman *et al.* (5). The lithium content in the serum was *ca.* 0.10 mEq/kg throughout the experiment.

The levels of phosphoinositides were estimated relative to normal control levels after the 1st and 3rd wk of lithium administration. The results are shown in Figure 1. There was no significant difference in PI, PIP or PIP_2 when compared with the normal levels in the brains at 1 wk and 3 wk after initiation of lithium treatment. This result is in conflict with that of a previous report (17), but in agreement with the result of another (18). Though the turnover rates of PI, PIP and PIP_2 were not determined, the rates of hydrolysis of inositol phospholipids seemed not to be significantly affected by lithium.

TABLE 1

Lithium Content in Rat Brain After the Indicated Days of Administration[a]

Days after start of Li	Number of rats	Li in brain (mEq/kg)
1	4	0.337 ± 0.11
3	6	0.321 ± 0.03
5	5	0.213 ± 0.03
7	3	0.256 ± 0.05
11	3	0.243 ± 0.03
15	3	0.293 ± 0.01
22	3	0.228 ± 0.02

[a]Three-week-old male Spraque-Dawley rats were administered lithium carbonate at 2.7 mEq Li_2CO_3/kg/d. At 24 h after administration, the rats were decapitated and the brains were removed immediately. Lithium content was estimated by atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, CT, Model 703). Values represent the mean ± SEM.

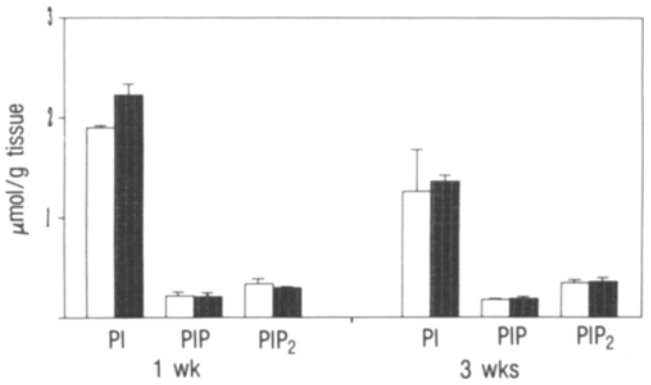

FIG. 1. The effect of lithium on the levels of inositol phospholipids in rat brain. Open bars, control; gray bars, Li-treated. The methods for estimation of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) in rat brain homogenates are described in Materials and Methods. Results are mean ± SEM from 32 rats. Treated groups show no significant difference from the control by Student's two sample *t*-test, $P > 0.1$.

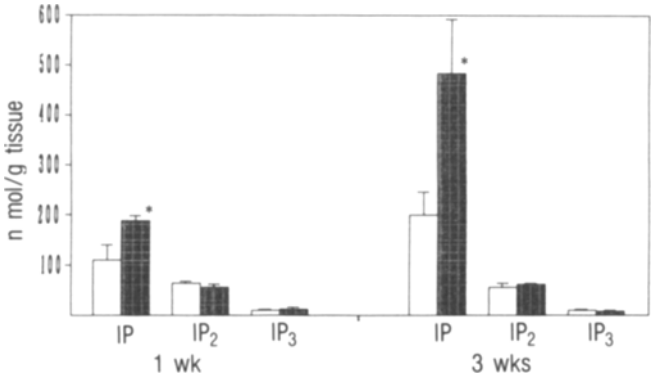

FIG. 2. The effect of lithium on the levels of inositol phosphates (IP) in rat brain. Open bars, control; gray bars, Li-treated. IP, inositol diphosphate (IP_2) and inositol triphosphate (IP_3) were analyzed in rat brain homogenates as described in Materials and Methods. The different control levels of IP at 1 wk and 3 wk seem to reflect the developmental states of 4- and 6-wk-old rats. Results are mean ± SEM from 32 rats. Statistical significance is indicated by *, $P < 0.01$ (Student's two sample *t*-test).

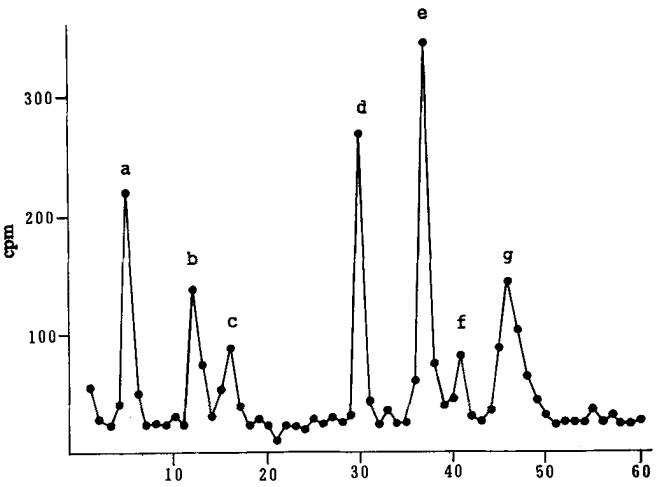

FIG. 3. Fractionation of radioactive inositol phosphates on Partisil SAX high-performance liquid chromatography. a: Inositol; b: inositol 1-phosphate; c: inositol 4-phosphate; d: inositol 1,4-diphosphate; e: inositol 1,3,4-triphosphate; f: inositol 1,4,5-triphosphate; g: inositol 1,3,4,5-tetraphosphate. The details of the experiment are described in Materials and Methods.

Furthermore, we tested the effect of lithium on the IP, IP_2 and IP_3 contents of the rat brains (Fig. 2). The level of I-1-P rose about 1.7-fold from 109 ± 29.2 to 187 ± 9.7 nmol/g tissue in rats after 1 wk of lithium administration and about 2.4-fold from 199 ± 46.3 to 483 ± 108.6 nmol/g tissue after 3 wk of lithium administration. An increase in I-1-P by chronic lithium treatment of the rats for 10 d was also observed by Sherman *et al.* (5). IP_2 showed no significant change (Fig. 2) in response to lithium.

The level of IP_3 at 1 wk was increased from 9.7 ± 1.4 nmol/g tissue to 11.3 ± 3.8 in the lithium group, while at 3 wk, it was decreased from 10.4 ± 0.4 to 7.9 ± 1.0 nmol/g tissue. However, the apparent decrease of IP_3 upon chronic administration of lithium was not statistically significant (Student's *t*-test, $P > 0.1$).

Next, IP_3 was fractionated into I-1,3,4-P_3 and I-1,4,5-P_3 by HPLC (Fig. 3). As shown in Figure 3, the separation of these two IP_3 was satisfactory. I-1,3,4-P_3 accounted for 95.8 ± 2.7% of IP_3 in the control. On the other hand, after 3 wk of lithium administration, the proportion of I-1,3,4-P_3 in IP_3 was decreased to 69.6 ± 11.8%. Our experiments thus showed that the proportion of I-1,4,5-P_3 in IP_3 was increased from 4.2 to 30.4% of total IP_3 in the chronically lithium-administered rat brains, although the total quantity of IP_3 was not significantly altered (Fig. 4).

No significant amounts of IP_4 were detected in the normal or lithium-treated rat brains.

DISCUSSION

Lithium carbonate was given orally to rats, mimicking the route by which patients with affective disorders are treated. The dose of 100 mg/kg/d of lithium carbonate, amounting to 2.7 mEq/kg/d, was a little smaller than that used by other authors, such as Sherman *et al.* (5)

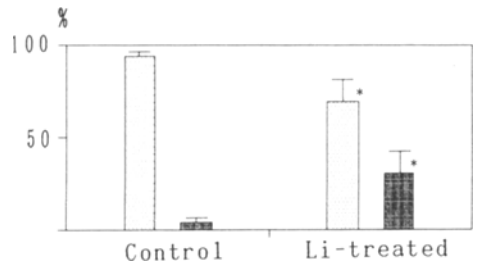

FIG. 4. The effect of lithium on the inositol triphosphate composition. Dotted bars: inositol 1,3,4-triphosphate (1,3,4-P_3); gray bars: inositol 1,4,5-triphosphate (1,4,5-P_3). I-1,3,4-P_3 and I-1,4,5-P_3 were fractionated on a Partisil SAX high-performance liquid chromatography column as described in Materials and Methods. Inositol phosphates were digested with alkaline phosphatase and analyzed by gas–liquid chromatography as described in Materials and Methods. Results are mean ± SEM from 24 rats. Statistical significance is indicated by *, $P < 0.01$ (Student's two sample *t*-test).

(3.6 mEq/kg/d). Nevertheless, the distribution and concentrations of lithium in the rat were in excellent agreement with the results of Sherman *et al.* (5). Further, our routine clinical experience suggested that this dose is sufficient for the effective long-term treatment of patients with affective disorders (19).

Initially, we measured the contents of phosphoinositides in the rat brains after 1 wk and 3 wk of lithium administration as compared with those of normal rats and found no significant differences in the quantities of PI, PIP and PIP_2. Previous studies on inositol phospholipid levels have yielded inconsistent results. Joseph *et al.* (17) estimated a decrease in PI from 3.8 to 2.9% in lithium-administered rat brain, while Honchar *et al.* (18) found no significant change. Although the turnover rates of PI, PIP and PIP_2 were not estimated, our data are in agreement with those of Honchar *et al.* (18). We intend to examine the metabolic rates of all inositol phospholipids as a next step for clarifying the lack of a decrease of PI, PIP and PIP_2.

When we estimated the quantities of IP, IP_2 and IP_3, we found that the quantity of IP rose about 1.7 times after 1 wk and about 2.4 times after 3 wk of lithium administration compared with the levels of normally fed rats. Renshaw *et al.* (20) and Honchar *et al.* (18) reported inconsistent results concerning the effect of lithium ions on IP phosphatase. Renshaw *et al.* (20) observed a doubling of IP phosphatase activity after 24 d of lithium administration to rats, while Honchar *et al.* (18) found no significant increase in this enzyme in rat cerebral cortex. This discrepancy is rather difficult to explain. However, our experiments clearly showed a remarkable accumulation of IP with chronic lithium administration (see Fig. 2).

Then we fractionated IP_3 by HPLC into I-1,3,4-P_3 and I-1,4,5-P_3. Meek (21) had reported that in normal rat brain, I-1,4,5-P_3 accounted for 80.5% of IP_3 after decapitation, but our results showed that 95.8% of IP_3 was I-1,3,4-P_3. In platelets (22,23) and in pancreatic islets (24), most of the IP_3 is present as I-1,3,4-P_3, which is in accordance with our result. Our key finding was that after 21 d of lithium administration, the proportion of I-1,4,5-P_3 in IP_3 had increased from 4.2 to 30.3%. This is a 7.2-fold increase when compared with controls (Fig. 4). This increase in the physiologically active neurotransducer, I-1,4,5-P_3, may play a key role in the therapeutic action of lithium in patients with affective disorders.

Our finding that long-term administration of lithium increased I-1,4,5-P_3 and decreased I-1,3,4-P_3, while the total amount of IP_3 was unchanged, may imply that lithium inhibits the conversion of I-1,4,5-P_3 to I-1,3,4-P_3. Accumulation of I-1,4,5-P_3 inside nerve cells causes the release of calcium ions from the endoplasmic reticulum of the cells into the inside of the axon terminals (4), which in turn causes the release of neurotransmitters contained in the synaptic vesicles of the nerve ending into the synaptic gaps (25,26). On the other hand, I-1,3,4-P_3 has a much weaker ability (*ca.* 1/30) to mobilize the calcium ions from intracellular stores than does I-1,4,5-P_3 (27). Worley *et al.* (28) demonstrated the presence of high-affinity selective binding sites for I-1,4,5-P_3 in rat brain at a level 100–300 times higher than in other tissues. Moreover, they found heterogeneous localizations of the I-1,4,5-P_3 receptor in rat brain, with particularly high concentrations of binding sites in the molecular layer of the cerebellum, prefrontal cortex, hippocampus and the superficial layers of the cerebral cortex. This could explain why lithium is so effective in treating depressive as well as manic disorders. The role of the cortex is commonly believed to be the integration of sensory input information and modulation of output signals along the pathways (29,30). The lower basal ganglia of the brain, which influence emotions when stimulated directly (31), require integrative control from the upper cortex (32). Affective disorders may be attributed to disorders derived from the cerebrum, especially the prefrontal cortex, leading to failure to control the basal ganglia adequately. An increased I-1,4,5-P_3 level might help to amplify the signals in a disordered patient to levels similar to those in normal individuals. Thus, disturbed neurons may recover normal functions.

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Characterization of the Diacylglycerol Acyltransferase Activity in the Membrane Fraction from a Fungus

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In an attempt to clarify the mechanism of lipid accumulation in *Mortierella ramanniana* var. *angulispora*, diacylglycerol acyltransferase (DGAT) in the membrane fraction from this fungus was characterized. The enzyme had an optimum pH of 7.0–7.5, and enzyme activity was blocked by SH-reagents. Metal ions were not essential for maintaining DGAT activity. *n*-Octyl-β-D-glucoside, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and Tween 80 were found to preserve activity, while Triton X-100 and sucrose monolaurate inhibited it. As the inhibition of DGAT activity by Triton X-100 was overcome by the addition of diacylglycerol (DG), the dependency of DGAT activity on exogenous DG was determined in the presence of 0.1% Triton X-100. DGAT activity in the membrane fraction was traced in fungi cultured for different time periods or in media at different carbon to nitrogen (C/N) ratios. Although the increase in total lipid content with culture time was accompanied by an increase in DGAT activity, total lipid changes related to changes in C/N ratio did not correlate with DGAT activity. Factors other than DGAT activity in the membrane fraction would appear to be involved in the regulation of total lipid content in this fungus.
Lipids 28, 583–587 (1993).

The enzyme, diacylglycerol acyltransferase (DGAT) catalyzes the final step in triacylglycerol (TG) biosynthesis. Although the enzyme activity has been studied in animal cells (1), higher plants (2,3) and in microorganisms (4), the physiological role of DGAT still remains unclear. One problem is to determine which enzyme is the rate-limiting step in TG synthesis, as DGAT (5–7) and phosphatidate phosphohydrolase (8–10) have been shown to play a rate-limiting role. Another problem is to show how DGAT contributes to the fatty acid composition of TG. Some reports have shown that DGAT has a broad acyl-donor specificity and that the fatty acid incorporated at *sn*-3 position is dependent on the fatty acid pool available to the enzyme (3,11). Other reports have shown that some fatty acids are preferentially incorporated into TG *via* DGAT in plants (12,13).

Mortierella genus has been found to have a high lipid content and to contain polyunsaturated fatty acids, such as γ-linolenic acid (14–16). γ-Linolenic acid production by the fungus is so efficient that it has become an industrial method (14). Previous studies have characterized the fatty acid incorporation into the lipids of *Mortierella* genus (17,18). It was shown that the fatty acids were effectively incorporated into TG of this fungus. Fatty acid incorporation into TG may be partly mediated *via* phospholipids, such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, as well as phosphatidic acid. DGAT is unique to TG biosynthesis; hence the properties of DGAT and the possible involvement of DGAT in the lipid accumulation in this fungus were examined.

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; C/N ratio, carbon to nitrogen ratio; DG, diacylglycerol; DGAT, diacylglycerol acyltransferase; EDTA, ethylenediaminetetraacetic acid; TG, triacylglycerol; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. [1-^{14}C]Oleoyl-CoA (58 mCi/mmol) was obtained from New England Nuclear Corporation (Boston, MA). Oleoyl-CoA was purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100 and Tween 80 were from Nacalai Tesque (Kyoto, Japan). *n*-Octyl-β-D-glucoside and 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS) were from Dojin Pure Chemical Industries (Kumamoto, Japan). Sucrose monolaurate was obtained from Mitsubishi-Kasei Food Corp. (Tokyo, Japan). Silica gel G thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Microorganisms. Mortierella ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute of Fermentation (Osaka, Japan). The fungi were maintained on a yeast-extract/malt-extract agar medium. The liquid medium was basically as described previously (15), *i.e.*, it contained 30 g glucose, 1.5 g $(NH_4)_2SO_4$, 3 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 5 g $CH_3COONa \cdot 3H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1.2 mg $CaCl_2 \cdot 2H_2O$, 0.2 mg $CuSO_4 \cdot 5H_2O$, 1.0 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 2 mg thiamine-HCl and 0.02 mg biotin in 1 L of distilled water (pH 5.7). Cultures were grown in rotary shakers (180 rpm) at 30°C for specified periods. The carbon to nitrogen (C/N) molar ratio was 38. Different C/N ratios were attained by varying the amount of $(NH_4)_2SO_4$ added to the medium.

Preparation of membrane fractions. Fungal cells (100–300 mg dry cell weight) suspended in 20 mL of 10 mM phosphate buffer (pH 7.0) containing 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA) were homogenized with a Braun Homogenizer (Melsungen, Germany) together with 20 mL of glass beads (0.45–0.50 mm diameters). The homogenates were centrifuged at 1500 × *g* for 5 min to remove cell debris and nuclei. The supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet thus obtained was dissolved in 10 mM phosphate buffer (pH 7.0) containing 0.25 M sucrose, and again centrifuged at 100,000 × *g* for 1 h. The pellet was suspended in 10 mM phosphate buffer (pH 7.0) containing 0.25 M sucrose, and all suspensions were stored at −20°C until use. This membrane fraction served as the enzyme source.

DGAT assay. The assay mixture contained 10 mM phosphate buffer (pH 7.0), 150 mM KCl, 3.4 μM (0.2 μCi/mL) [1-^{14}C]oleoyl-CoA, 1 mM 1,2-diolein, 0.1% Triton X-100 and appropriate amounts of the membrane fraction in a final volume of 100 μL. In initial experiments, no 1,2-diolein or Triton X-100 was added to the reaction mixture. When necessary, 10 mM acetate buffer or 10 mM Tris buffer was used to adjust the pH of the assay mixture. The

reaction was carried out at 30°C for 5 min unless otherwise stated, and was stopped by the addition of 3 mL of chloroform/methanol (1:2, vol/vol). Lipids were extracted by the method of Bligh and Dyer (19) and separated by TLC on Silica gel 60 plates with benzene/diethyl ether/ethanol/NH_3 (50:40:2:0.5, by vol) as the first solvent, and hexane/diethyl ether (94:6, vol/vol) as the second solvent for developing the plates from the bottom up both times in the same direction (20). Radioactivity in TG was determined with a Beckman (Fullerton, CA) liquid scintillation system (model LS1701) with automatic quenching correction.

Other methods. Dry cell and total lipid content were measured and expressed in weight units as described previously (15). Protein was measured by the method of Bradford (21). The amount of each lipid class was determined from its fatty acid content measured by gas-liquid chromatography using heptadecanoic acid methyl esters as internal standards (17).

RESULTS AND DISCUSSION

In the present study we characterized DGAT activity in membrane fractions of *Mortierella ramanniana* var. *angulispora*. Although DGAT has been extensively studied in mammalian and plant cells, information on DGAT in fungi is quite limited.

The time course of radioactive TG formation from exogenous [1-^{14}C]oleoyl-CoA was measured in the membrane fraction (Fig. 1). The TG formation was linear for 5 min when using at least 20 μg protein. Figure 1 also shows that TG formation is dependent on the amount of protein at a fixed time of 5 min. The optimum pH for the DGAT activity was 7.0–7.5, this being essentially the same as the optimum pH range previously reported (2,22,23). The effect of oleoyl-CoA concentration on the incorporation of oleate from [1-^{14}C]oleoyl-CoA into TG is shown in Figure 2. Substrate saturation was reached at about 200 μM.

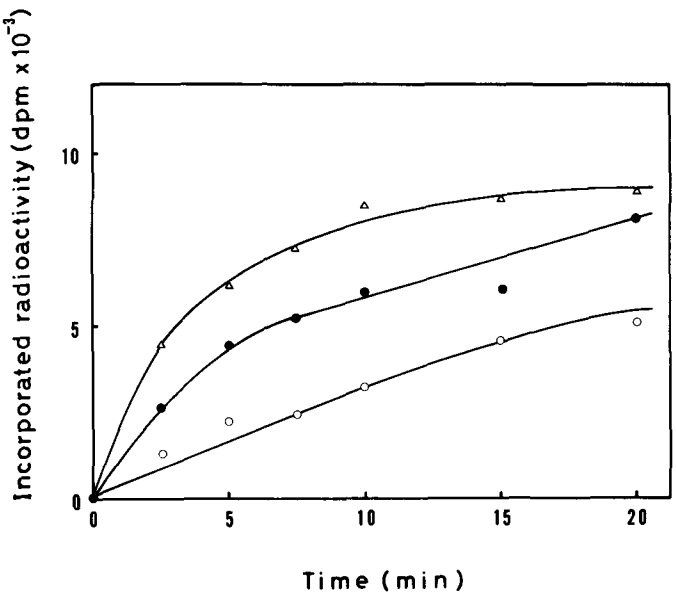

FIG. 1. Time course of the incorporation of oleate from [1-^{14}C]-oleoyl-CoA into triacylglycerol by a membrane fraction obtained from fungal cells cultured for 5 d. Membrane fractions containing 10 μg protein (○), 20 μg protein (●) or 40 μg protein (△) were incubated with [1-^{14}C]oleoyl-CoA for the time periods indicated. The assay mixture did not contain exogenous diacylglycerol or Triton X-100, as described in Materials and Methods. Data are presented as means of duplicates.

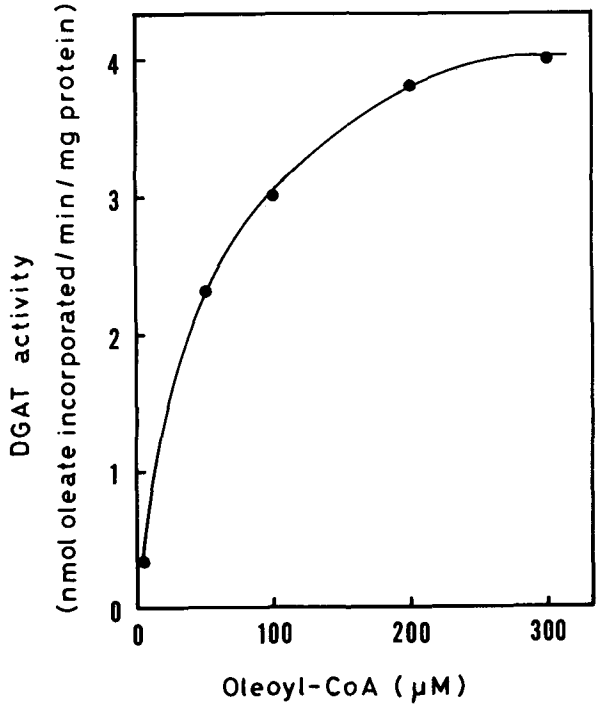

FIG. 2. Effect of oleoyl-CoA concentration on the incorporation of oleate from [1-^{14}C]oleoyl-CoA into triacylglycerol. Diacylglycerol acyltransferase (DGAT) activity was assayed using the membrane fraction (20 μg protein) which was obtained from fungal cells cultured for 5 d. The assay mixture did not contain exogenous diacylglycerol or Triton X-100 as described in Materials and Methods. Data are presented as means of duplicates.

The effects of reagents on DGAT activity are shown in Table 1. There was neither stimulation of DGAT activity by Mg^{2+} nor inhibition by EDTA. DGAT activity decreased by about one-half by the addition of SH-reagents, as previously described (24,25). Trifluoperazine, which caused a decrease in [1-^{14}C]fatty acid incorporation into TG in the intact fungal cells (18), did not change the

TABLE 1

Effect of Additives on Diacylglycerol Acyltransferase (DGAT) Activity in the Membrane Fraction from *Mortierella ramanniana* var. *angulispora*

Additives	DGAT activity[a] (% of control)
Mg^{2+} (5 mM)	104
EDTA (5 mM)	93
N-Ethylmaleimide (1 mM)	66
Dithiothreitol (1 mM)	45
Ethanol (2%)	102
Trifluoperazine (0.5 mM) in 2% ethanol	98

[a]The membrane fraction (20 μg protein) obtained from fungal cells cultured for 5 d was incubated for 5 min with [1-^{14}C]oleoyl-CoA without addition of exogenous diacylglycerol or Triton X-100. EDTA, ethylenediaminetetraacetic acid.

TABLE 2

Effect of Detergents on DGAT Activity in the Membrane Fraction from *Mortierella ramanniana* var. angulispora

Detergents		DGAT activity[a] (% of control)
n-Octylglucoside	(0.1%)	117
	(0.2%)	96
CHAPS	(0.05%)	128
	(0.1%)	72
Tween 80	(0.05%)	87
	(0.1%)	91
Triton X-100	(0.05%)	25
	(0.1%)	28
Sucrose monolaurate	(0.05%)	15
	(0.1%)	10

[a]The membrane fraction (20 μg protein) obtained from fungal cells cultured for 5 d was incubated for 5 min with [1-^{14}C]oleoyl-CoA without addition of exogenous diacylglycerol or Triton X-100. DGAT, diacylglycerol acyltransferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonion]-1-propanesulfonate.

activity. This is consistent with the interpretation that phospholipid acylation, rather than diacylglycerol (DG) acylation, is affected by trifluoperazine, which eventually leads to inhibition of TG biosynthesis.

While searching for a suitable detergent for solubilizing the enzyme, the effects of various detergents on DGAT activity were tested (Table 2). *n*-Octyl-β-D-glucoside, CHAPS and Tween 80 did not inhibit DGAT activity, whereas Triton X-100 and sucrose monolaurate greatly inhibited this activity. Inhibition of DGAT activity by Triton X-100 has been reported for preparations from higher plants (2,3). DGAT activity from rat liver has been solubilized with Triton X-100 (26) and DGAT from soybean cotyledons with CHAPS (27).

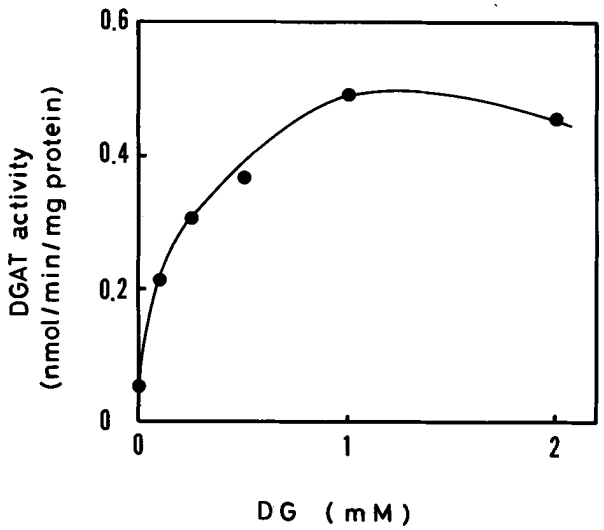

FIG. 3. Diacylglycerol (DG) dependency of DG acyltransferase (DGAT) activity in the presence of 0.1% Triton X-100. DGAT activity was assayed using a membrane fraction (20 μg protein) which was obtained from fungal cells cultured for 5 d. The assay was done in the presence of 0.1% Triton X-100 with increasing concentrations of exogenous 1,2-diolein as described in Materials and Methods. Data are presented as means of duplicates.

In the DGAT assay systems used, DGAT activity could not be shown to be dependent on exogenously added 1,2-diolein, possibly because exogenous DG had no access to the enzyme. This interpretation is consistent with the results of other studies in which it was difficult to show that exogenous DG was a substrate of the enzyme (2,24). Neither could DG dependency of the enzyme activity be demonstrated when DG was administered as ethanol solution. However, DG dependency of DGAT could be demonstrated in the presence of detergents (28).

As is evident from Table 2, Triton X-100 and sucrose monolaurate inhibited DGAT activity. To determine whether exogenous DG could overcome the inhibition of DGAT by Triton X-100, DGAT activity was measured at various concentrations of 1,2-diolein in the presence of 0.1% Triton X-100 (Fig. 3). Figure 3 shows that DGAT

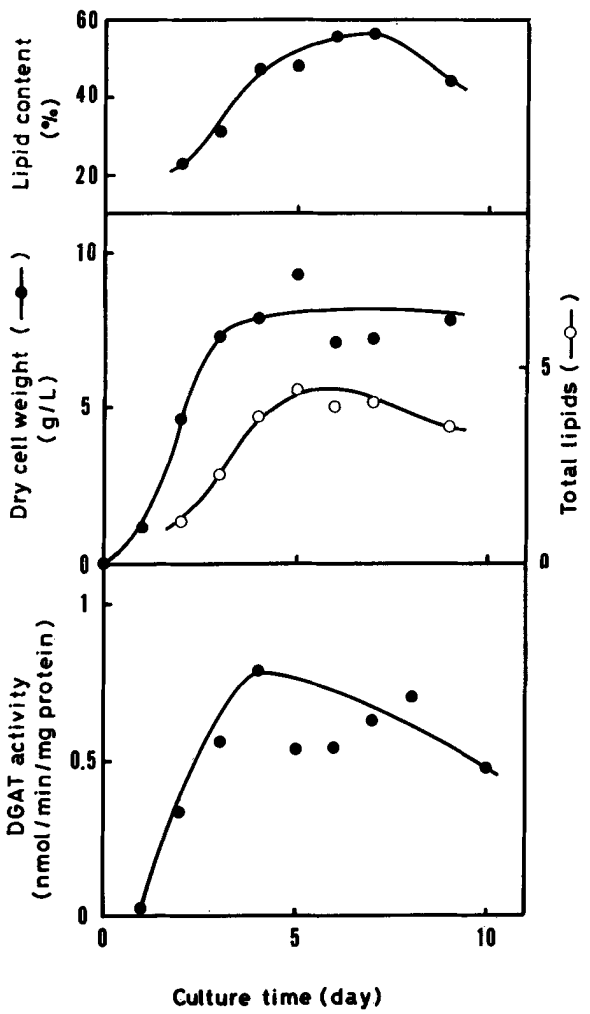

FIG. 4. Diacylglycerol acyltransferase (DGAT) activity in the membrane fraction and lipid accumulation during culture. DGAT activity shown in the lower panel was assayed for 5 min in the presence of 0.1% Triton X-100, 1 mM 1,2-diolein and 3.4 μM (0.2 μCi/mL) [1-^{14}C]oleoyl-CoA using a membrane fraction (20 μg protein) obtained from fungal cells cultured for the time periods indicated (means of duplicates). Triacylglycerol formation was linear for 5 min in all the assays. Dry cell weights (●) and total lipids (○) from fungal cells cultured for the indicated time periods are shown in the center panel (means of duplicates). Lipid content (total lipids/dry cell weight) is shown in the upper panel (means of duplicates).

activity increased with the amount of exogenous DG added and reached a plateau at 1 mM 1,2-diolein. This could be interpreted to mean that Triton X-100 disperses both endogenous and exogenous DG and makes them accessible as substrates. To improve the DGAT assay so that DGAT activity would not be dependent on the amount of endogenous DG in the membrane fraction, 0.1% Triton X-100 and 1 mM 1,2-diolein were therefore included in the assay system.

The effect of culture time on DGAT activity is shown in Figure 4. Fungal cells proliferated throughout the incubation period of 5 d. Lipid synthesis followed with a slight delay. The lipid content measured as total lipids/dry cell weight was highest in the early stationary growth phase, as is typical for lipid accumulation in oleaginous fungi (29). Lipid accumulation, which is associated with increased conversion of carbon substrate into lipids, may be partly due to the depletion of nitrogen in the media (29). Figure 4 shows that DGAT activity increased with culture time, which was accompanied by an increase in total lipid content, which peaked at day 4. The increase in total lipid content was primarily due to TG formation (Table 3), suggesting that DGAT activity could be related to the amount of TG produced. DGAT activity in the soluble fraction (supernatant of 100,000 × *g* centrifugation) on day 5 was 8.4% of the DGAT activity in the membrane fraction on day 5, indicating that membrane-bound DGAT activity was essential to TG formation in this fungus.

To shed light on the possible relationship between DGAT activity and lipid accumulation, DGAT activity under modified culture conditions that could affect lipid accumulation was also followed. When the C/N ratio of the culture medium was increased, which is known to affect lipid accumulation (29–31), it also resulted in lipid accumulation in our system (Fig. 5). The increase in C/N ratio in the medium also changed DGAT activity. However, the pattern of changes in lipid content did not necessarily correlate with DGAT activity, indicating that the relationship is not necessarily direct, but seems more complex.

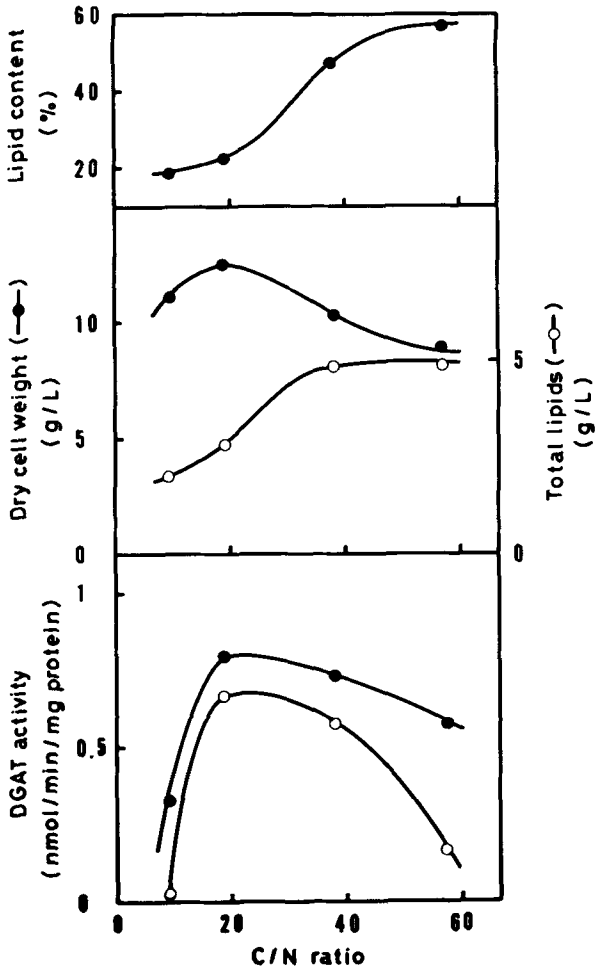

FIG. 5. Effect of carbon to nitrogen (C/N) ratio on diacylglycerol acyltransferase (DGAT) activity in the membrane fraction and on lipid accumulation. DGAT activity shown in the lower panel was assayed for 5 min in the presence of 0.1% Triton X-100, 1 mM 1,2-diolein and 3.4 μM (0.2 μCi/mL) [1-^{14}C]oleoyl-CoA using a membrane fraction (20 μg protein) obtained from fungal cells cultured for 4 d (●) or 5 d (○) at the indicated C/N ratios (means of duplicates). Triacylglycerol formation was linear for 5 min in all the assays. Dry cell weight (●) and total lipids (○) from fungal cells cultured for 5 d at the indicated C/N ratio are shown in the center panel (means of duplicates). Lipid content (total lipids/dry cell weight) is shown in the upper panel (means of duplicates).

TABLE 3

Changes in Lipid Class Distribution as Function of Culture Time in *Mortierella ramanniana* var. *angulispora*

Lipids[b]	Amount of each lipid class[a] (μmol/g dry cell weight)		
	1 Day	5 Days	8 Days
TG	83.7 (55.6%)	330.3 (82.8%)	344.6 (82.2%)
DG	5.7 (3.8%)	30.6 (7.7%)	31.2 (7.4%)
SE	8.1 (5.3%)	7.1 (1.8%)	13.0 (3.1%)
FFA	4.3 (2.8%)	5.3 (1.3%)	4.8 (1.1%)
PC	13.3 (8.8%)	6.6 (1.6%)	6.8 (1.6%)
PE	18.6 (12.4%)	8.1 (2.0%)	7.5 (1.8%)
PS	4.7 (3.1%)	2.1 (0.5%)	1.8 (0.4%)
PI	2.2 (1.5%)	2.4 (0.6%)	2.8 (0.7%)
GL[c]	10.1 (6.7%)	6.9 (1.7%)	6.9 (1.6%)
Total	150.8	399.0	419.7

[a]Values for 1 d and 8 d were calculated from data previously reported by us (Ref. 18). Values are expressed as means of triplicates.
[b]TG, triacylglycerol; DG, diacylglycerol; SE, steryl ester; FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; GL, glycolipids. Free sterol was also detected in the fungal lipids.
[c]The GL fraction was composed of a major unidentified GL and several minor fractions. The amount of GL was calculated on the assumption that GL had one fatty acid moiety.

In the present study, a crude membrane fraction served as the enzyme source. The amount of lipid in the membrane fraction was 1.5 ± 0.5 mg lipids/mg protein (n = 3). TG and DG amounted to about 70 and 7% of total lipids, respectively, as estimated by TLC densitometry (32). Taken together, it appears that DGAT activity in the membrane fraction does not directly correlate with accumulation of TG in the fungus. How DGAT activity is regulated by membranes rich in neutral lipids will still need to be determined.

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Lipid Biosynthesis in Cultured Arterial Smooth Muscle Cells Is Related to Their Phenotype

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During the atherogenic process *in vivo*, arterial smooth muscle cells (SMC) undergo changes in their phenotype. In the present study, rat SMC from primary cultures and from subcultures before 10 and after 200 passages, showing contractile-like, synthetic and transformed phenotypes, respectively, were compared in regard to their lipid content and biosynthesis. The rationale for comparing these phenotypes rests in the similar changes in phenotype of SMC that occur in the formation and progression of atherosclerotic lesions. Phenotype changes were shown to be associated with changes in the phospholipid content of SMC. Phospholipid levels increased, but not as significantly as did cholesterol levels when passing from contractile to synthetic and transformed cells (1.23 ± 0.18, 2.28 ± 0.26 and 3.25 ± 0.23 $\mu g/10^6$ cells, respectively). Cholesterol normalized in respect to cell protein was increased to the same extent. Lipid synthesis as judged by [^{14}C]acetate incorporation was increased 3- to 12-fold in the synthetic and transformed cells, respectively, compared to contractile cells. After thin-layer chromatography, radioactivity was shown to be markedly increased in most of the lipid fractions, but label in the cholesterol fraction of synthetic and transformed cells was increased by 7- and 21-fold, respectively. Thus, SMC *in vitro* were shown to drastically increase cholesterol biosynthesis associated with phenotype changes. Such changes are known to occur *in vivo* and might represent a critical step in the deposition of excess cholesterol within foam cells.
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Arterial smooth muscle cells (SMC) play a key role in atherogenesis (1-4). During the initial stages of the process, SMC migrate into the intima, proliferate and produce abundant extracellular matrix components (5,6). Later on, SMC are transformed into foam cells by accumulating lipids. These processes are conditioned by the change in the SMC phenotypic state (7,8). The adult contractile phenotype "dedifferentiates" into a synthetic phenotype expressed in both atherogenic and remodeling processes. Conversely, during the normal organogenesis, the fetal "primary" synthetic SMC differentiate into a contractile state in adult artery (9). Arterial SMC *in vitro* undergo similar changes from the contractile-like to the synthetic state, which is characterized by changes in cytoskeletal and cytoenzymatic features (5,7,10).

Because an important characteristic of the foam cell transformation *in vivo* is the accumulation of cholesterol, it seems of interest to determine whether the *in vitro* transformed SMC would exhibit similar characteristics. The lipid metabolism of cultured SMC has previously been reported to be related to regulating factors, such as atherogenic lipoproteins (11,12), eicosanoids (13) or tumor necrosis factor (14). Only a few studies have correlated SMC in the contractile or synthetic state with changes in lipid metabolism (11,13). Rat aortic SMC have been shown to generate a transformed phenotype which expresses tumorigenic potential as well as some similarities with rapidly proliferating cells (15). This transformed cell model is remarkable in the light of the transforming elements detected in arteriosclerotic plaques (16), and accounts for Benditt's atherogenic monoclonal hypothesis (17).

The aim of the present study was to compare the phospholipid and cholesterol contents and lipid biosynthesis in "contractile-like," "synthetic" and "transformed" SMC phenotypes in culture.

MATERIALS AND METHODS

General. [U-^{14}C]Acetic acid, sodium salt (95mCi/mmol) was purchased from the Commissariat à l'Energie Atomique (Saclay, France). Minimum essential medium (MEM) with Earle's salts, antibiotics and L-glutamine were obtained from BioMèrieux (Marcy l'Etoile, France). Calf serum and trypsin were from GIBCO (Life Technologies Ltd., Paisley, Scotland). Silica gel plates (G60, 20 × 20 cm) were obtained from Merck (Darmstadt, Germany). Radioactivity was scanned with an Intertechnique liquid scintillation scanner, model SL 3000 (Kontron, St. Quentin Yvelines, France). Cells were counted in a Coulter counter (ZBI Coulter, Coultronics, Margency, France). Protein content was measured according to Lowry *et al.* (18).

Cell culture. Aortic SMC were obtained from 6- to 10-week-old rats (19). Thoracic aorta was dissected and SMC dispersed into single cells by collagenase (2 mg/mL, CLSI from Worthington Biochemical Corp., Freehold, NJ) and elastase (0.4 mg/mL, type III from Biosys, Compiègne, France). Cells were seeded in Petri dishes (35 mm) or 6-well plates ($3-5 \times 10^4$ cells/cm^2). Cells were grown in MEM medium supplemented with 10% newborn calf serum, penicillin/streptomycin (100 units/mL and 50 μg/mL, respectively) and glutamine (2 mM). Medium was renewed three times a week. Secondary cultures were initiated after either low or high passages using trypsin (0.08%).

SMC *in vitro* showed numerous morphological and functional changes characteristic of the modulation from the contractile to the synthetic phenotype. Cells in primary culture (Fig. 1A) exhibited "contractile-like" properties. Thus, they were considered as the contractile SMC model of normal adult aorta. Subcultured vascular SMC from the second to the seventh passage (Fig. 1B) displayed the same "synthetic state" as SMC from the diffuse intimal thickening or the embryonic aorta (9,10). They retained several important features of vascular SMC *in situ, i.e.,* a well-developed microfilament system, vimentin-type intermediate filaments, desmin and α-actin cytoskeletal markers. In the present study, synthetic state cells at 5-10 passages were used. The third SMC model described as "transformed phenotype" was obtained from two

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Abbreviations: 1,2-DG, 1,2-diacylglycerols; 1,3-DG, 1,3-diacylglycerols; FFA, free fatty acids; HMG-CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; MEM, minimum essential medium; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TG, triacylglycerols.

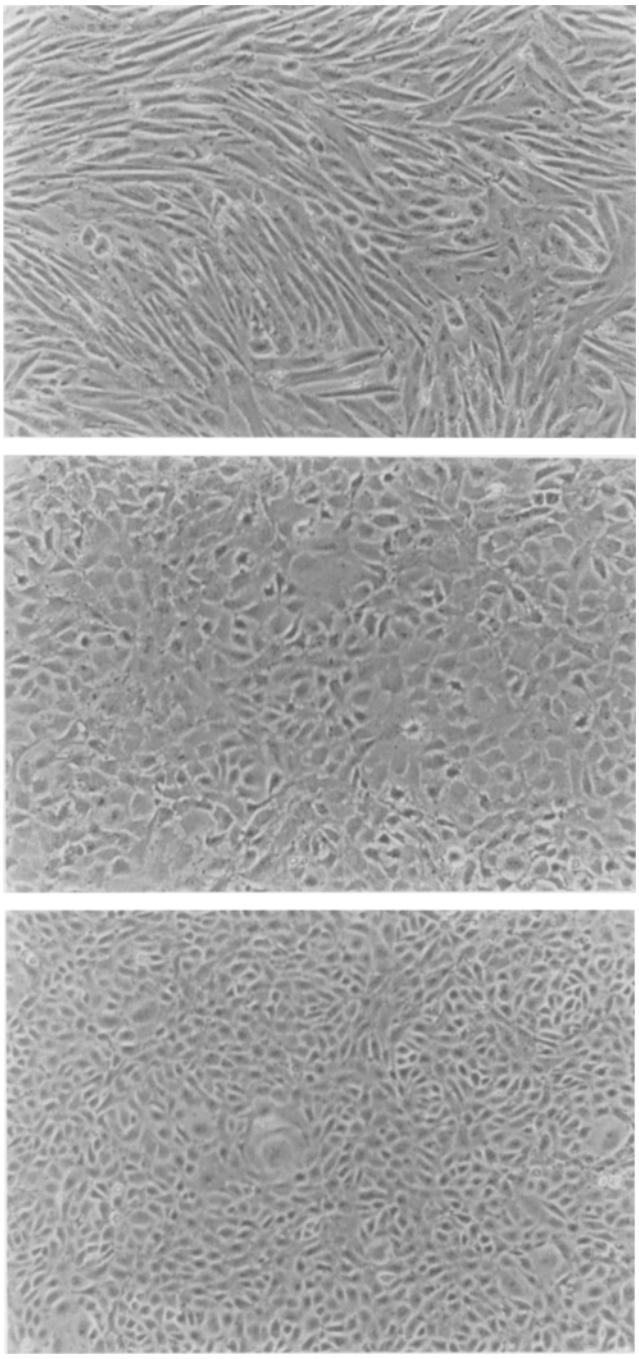

FIG. 1. Phase-contrast micrographs of the three cell phenotypes, exhibiting mono- and multi-layered areas ("hill and valley pattern"), typical of arterial smooth muscle cell growth (Refs. 1,15). At confluency, the elongated "contractile-like" cells in primary cultures (A) are clearly distinct from the spreaded "synthetic" cells of secondary cultures (Passage 6, B) and packed "transformed" cells displaying scattered giant cells (Passage 215, C). Magnification, ×229.6.

established cell lines originating from high-passaged rat aortic SMC (Fig. 1C). In addition to their synthetic features, these two cell lines exhibit immortalized and tumorigenic properties (15). The cells presently used were over 200 passages old.

Cholesterol and phospholipid analysis. Cells were sampled in the confluent state, after trypsinization and cell counting. The cell numbers in contractile, synthetic and transformed cells were 175 ± 11, 170 ± 19 and $257 \pm 16 \times 10^3$ cells/cm^2, respectively. Lipids were extracted according to a modification of the Folch *et al.* (20) method: 1 mL of the cell suspension was added to 25 mL of dichloromethane/methanol (2:1, vol/vol) and 5 mL of NaCl. After decantation, the organic phase was evaporated. Total phospholipids were assayed by the colorimetric method according to Stewart (21). Free cholesterol was assayed using the Omodeo-Sale *et al.* (22) method.

Cholesterol biosynthesis. At confluence, SMC cultures from the three different phenotypes were labeled during 60 min with [U^{14}C]acetic acid (2 μCi/mL) in MEM + 10% newborn calf serum. Lipids were extracted by the modified Folch method and analyzed. The radioactivity of the total lipid fraction was measured on a sample of the lipid extract (20 μL), which was added to 8 mL of scintillating mixture composed of 4 g of diphenyloxazole (J.T. Baker Chemicals, Deventer, Holland), 0.2 g of 1,4 di(2-(5phenyloxazolyl)benzene) (Koch Light Laboratories Ltd., Colnbrook, Bucks, England) and toluene (1000 mL).

The different lipid fractions were separated according to Kunz (23) by one-step one-dimensional thin-layer chromatography using silica gel plates. After development, the plates were air-dried and the lipid fractions were stained with I_2. Radiolabelled fractions were detected by autoradiography (Film Kodak, XRay, Eastman Kodak Co., Rochester, NY). Silica gel bands were scraped off and radioactivity was quantitated by liquid scintillation counting.

Statistical analysis. The significance of differences between the SMC phenotypes were computed using the unpaired Student's *t*-test. Results are presented as means ± SEM.

RESULTS

The different phenotypes (contractile-like, synthetic and transformed cells) exhibited three distinct morphological patterns (Fig. 1). In contractile cells, the protein mass seemed mainly associated with the myofilament compartment (Fig. 1A), whereas there were large amounts of rough endoplasmic reticulum in synthetic and transformed cells (Fig. 1B and 1C). The cholesterol content of contractile-like SMC was extremely low (1.23 ± 0.18 μg/10^6 cells). It increased approximately two- to threefold in synthetic and transformed cells, respectively (Fig. 2). The contractile-like cells were richer in phospholipids (8.0 ± 0.5 μg/10^6 cells, Fig. 2) than in cholesterol. The phospholipid content was moderately increased to 10.1 ± 0.9 and 13.2 ± 1.7 μg/10^6 cells in synthetic and transformed cells, respectively (Fig. 2). This increase was significant only in transformed SMC ($P < 0.05$). The protein content of contractile, synthetic and transformed cells was 53 ± 3.7, 58.7 ± 7.5 and 55 ± 3.8 μg/10^6 cells, respectively ($n = 6$). When cholesterol was normalized in respect to the cell protein content, the cholesterol/protein ratio was increased 1.7-fold in synthetic and 2.6-fold in transformed cells, *vs.* 1.8- and 2.7-fold, when normalized in respect to cell number as shown in Figure 2.

Lipid synthesis followed by [^{14}C]acetic acid incorporation into total lipids was particularly low (2236 ± 362

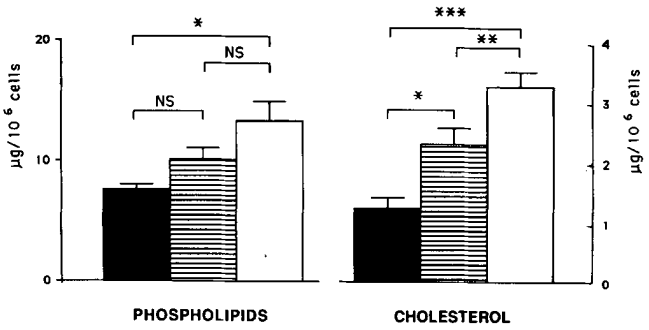

FIG. 2. Changes in free cholesterol and phospholipid content in contractile-like (full bars, n = 7), synthetic (hatched bars, n = 7) and transformed (open bars, n = 8) smooth muscle cell phenotypes in culture. Values are means ± SEM. Significance: $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***). NS, not significant.

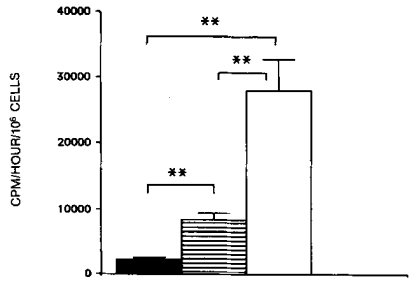

FIG. 3. [^{14}C]Acetic acid incorporation into the total lipids of contractile-like (full bars, n = 5), synthetic (hatched bars, n = 11) and transformed (open bars, n = 12) smooth muscle cell phenotypes in culture. Values are means ± SEM. Significance: $P < 0.01$ (**).

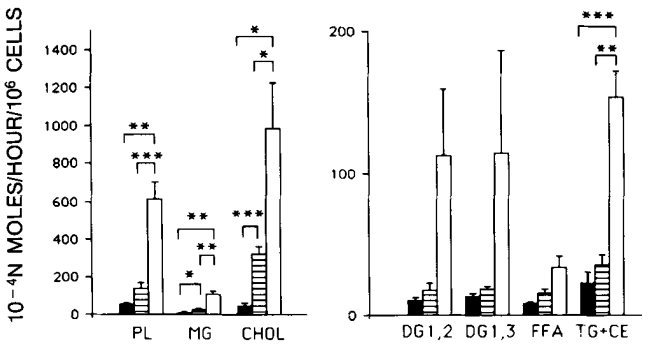

FIG. 4. [^{14}C]Acetic acid incorporation into different lipid fractions of contractile-like (full bars, n = 5), synthetic (hatched bars, n = 11) and transformed (open bars, n = 12) smooth muscle cell phenotypes in culture. PL, phospholipids; MG, monoacylglycerols; CHOL, cholesterol; DG 1,2, 1,2-diacylglycerols; DG 1,3, 1,3-diacylglycerols; FFA, free fatty acids; TG + CE, triacylglycerols + cholesteryl esters. Values are means ± SEM. Significance: $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

cpm/h/10^6 cells) in slowly proliferating contractile-like SMC (Fig. 3). Total lipid synthesis was increased threefold in the synthetic SMC ($P < 0.01$) and twelvefold in the transformed cells ($P < 0.01$). Lipid synthesis associated with various lipid fractions, namely mono- and diacylglycerols, phospholipids, fatty acids, free and esterified cholesterol (+ triacylglycerols), was low in contractile-like cells, but was much greater in actively proliferating modulated SMC (Fig. 4). In transformed cells, the biosynthesis was increased 12-fold for phospholipids ($P < 0.01$), 21-fold for free cholesterol ($P < 0.05$) and 7-fold for cholesteryl esters and triacylglycerols ($P < 0.001$). In synthetic SMC, [^{14}C]acetate incorporation was enhanced 7-fold in the free cholesterol fraction. In the present experiments, [^{14}C]-acetate incorporation predominantly occurred into the free cholesterol fraction, which reached 25.4% (±6.6) in contractile-like cells, but 51.7% (±3.4) and 42.6% (±3.5) in synthetic and transformed cells, respectively.

DISCUSSION

The present study clearly indicates that cultured SMC of different phenotypes, namely contractile, synthetic and transformed, exhibit marked differences in their lipid content, especially cholesterol, apparently owing to a large increase in lipid biosynthesis. Of interest is that an *in vivo* phenotype modification of SMC in arteries, similar to that observed here in rat aortic cultures after numerous passages, seems to be one of the primary events in atherogenesis (1-3,5,7). A spontaneous change in cell phenotypes was also observed in long-term culture SMC lines originating from bovine carotid artery (24). Of additional interest is that in SMC in atherosclerotic plaques, it is also cholesterol and cholesteryl esters that primarily accumulate (25), rather than phospholipids. Therefore, it would seem that the accumulation of cholesterol shown in SMC, without increased levels of cholesterol in its environment, could result from the proliferation and transformation of these cells, at least as concluded from the present *in vitro* experiments on rat aortic SMC.

To date, very few studies have examined the consequences of *in vitro* SMC dedifferentiation on cellular lipid metabolism (11-13). The present findings in rat confirm the previous work of Tabacik *et al.* (26) in SMC from rabbits that indicated that cholesterol biosynthesis was markedly increased in synthetic cells compared to contractile cells. Nevertheless, near the end of the culture life, *i.e.*, at 16 passages, the authors observed a drastic decrease in cholesterol production that was not noted under our conditions. This could be explained either by the fact that we used cells before 10 or after 200 passages, but not at 16 passages, or that we used cell numbers as the quantitative reference instead of protein mass (26). Cellular and matrix proteins are known to be increased with increasing passages (4,8). For these reasons, as did Campbell *et al.* (11), the results have been expressed on the basis of cell number rather than cell protein, because of the marked changes that occur in the type of cellular organelles with changes in the SMC phenotype. Nevertheless, when cells passed from the contractile to the synthetic and the transformed states, normalization of cholesterol in respect to either cell protein or cell count showed a similar increase (1.7 and 2.6, *vs.* 1.8 and 2.7, respectively).

We noted a low turnover of triacylglycerols (TG) in contractile cells, as was observed in myotubes during myogenic differentiation, contrasting with a large store of TG in myoblasts (27). Thus, in spite of their different structure and function, synthetic SMC and mononucleate myoblasts seem to share enhanced neutral lipid accumulation. The diacylglycerol (DG) turnover was greater than that of TG in the synthetic and transformed phenotypes.

This could be explained by a stimulation of the phosphoinositol cycle under our culture conditions. This would be in accordance with the known activation of inositol triphosphate, DG and protein Kinase C, in response to platelet-derived growth factor (PDGF) (28). PDGF is expressed during the SMC phenotype changes and could be self-amplified in an autocrine way (1,5), particularly in the rapidly replicating SMC (4) and in the transformed cells (15).

A question that can be raised is whether the transformed SMC used in our studies have any relevance to the atherogenic SMC in human arteries. In this context, it has been postulated that only a small group of cells with proliferative properties enters into the formation of an atherosclerotic plaque (17). It seems that these selected cells, known to transform readily into foam cells, could have, in regard to cholesterol biosynthesis, properties similar to those of the transformed cells used in the present study, namely a huge increase in cholesterol biosynthesis. However, under our present experimental conditions, almost solely free cholesterol accumulated in the cultured SMC. By contrast, in atherosclerotic plaques, both free and esterified cholesterol can be found (25). However, it can be postulated that the formation of cholesterol esters depends on the environment, namely the supply of fatty acid and particularly oleate, as shown by previous investigators (12,29,30).

As to the mechanisms involved in the increased cholesterol biosynthesis, these may be related to several different processes. One of them may be increased cell proliferation. Both enhanced [^{3}H]thymidine incorporation and cholesterol levels have been observed in cultured aortic SMC from human atherosclerotic lesions (31), suggesting a close relationship between intracellular lipid accumulation and cell proliferation. In addition, it seems that it is mainly cholesterol biosynthesis that is associated with cell proliferation, since specific inhibitors of cholesterol synthesis reduce cellular proliferative activity (32). HMG-CoA reductase is known to be one of the first dysregulated enzyme in neoplasic proliferating cells. Another possibility is that the increase in the lipid mass of plasma membranes might be related to changes in size and shape of cultured cells (Fig. 1A, B, C). Moreover, cholesterol plays a role in the regulation of ionic pumps and the maintenance of enzymatic functional capacities (33). It has been shown that hydrolase enzymatic activities, such as acid phosphatase and esterase, are more expressed in proliferative SMC (10); cholesterol enrichment might influence this regulation. Finally, cholesterol accumulation in cells *in vitro* could depend on enhanced secretory activities of modulated SMC. Synthetic (5,8) and transformed (15) cells are known to show extensive intracellular membrane-rich endoplasmic reticula.

In conclusion, the results of the present study show that dedifferentiation in cultured arterial SMC, which is characterized by enhanced proliferative capacity, is associated with marked increases in cholesterol biosynthesis and cholesterol accumulation. As it is known that such modulated cells have an increased capacity to metabolize low-density lipoproteins and, particularly, very low density lipoproteins (11), the process could constitute one of the early events in the transformation of SMC into foam cells.

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The Esterified Plasma Fatty Acid Profile Is Altered in Early HIV-1 Infection

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Previous studies have shown that alterations in micronutrient utilization occur in patients with Acquired Immune Deficiency Syndrome. In this study, total plasma fatty acid composition was measured in 36 homosexual men infected with the Human Immunodeficiency Virus 1 (HIV-1) and in 17 HIV-1 seronegative homosexual men in order to evaluate differences associated with early HIV-1 infection. Immunologic assessment included CD4 cell number count and lymphocyte blastogenesis in response to the mitogens phytohemagglutinin (PHA) and pokeweed (PWM). The mean total amount of ω6 polyunsaturated fatty acids (18:2 and 20:4) was significantly lower in the HIV-1 seropositive subjects (38 ± 8.1% SD) as compared to HIV-1 seronegative subjects (43 ± 4.2%; $P = 0.0027$). This was also reflected in a higher level of total saturated fatty acids (16:0 and 18:0) in HIV-1 seropositive subjects (30 ± 2.2% *vs.* 26 ± 2.8%; $P = 0.0001$). The ratio of linoleic to arachidonic acid (18:2 to 20:4) was higher in the HIV-1 seropositive group (6.76 ± 4.88) compared to the HIV-1 seronegative group (4.86 ± 1.37; $P = 0.0213$). The response to PHA in seropositive subjects correlated inversely with total plasma ω6 fatty acids ($r = -0.36$; $P = 0.027$), and directly with the 18:2 to 20:4 ratio ($r = 0.33$; $P = 0.046$). CD4 cell counts and the response to PWM did not correlate with plasma fatty acid levels in HIV-1 seropositive subjects. We conclude that early HIV-1 infection is associated with lower plasma ω6 polyunsaturated fatty acids, notably arachidonic acid, than are controls, and that the changes in the plasma fatty acid profile correlate with some indices of immune function. ***Lipids 28,*** **593–597 (1993).**

The clinical course of infection with the Human Immunodeficiency Virus 1 (HIV-1) varies, but its manifestations include many symptoms or signs that may affect food intake and/or nutritional status (1). In addition, energy requirements may be increased by infectious processes. Oral intake may be adversely affected by mucosal lesions, and absorption may be limited by intestinal infections and other lesions which may lead to diarrhea and malabsorption. Drug treatments may interfere with nutrient absorption and/or metabolism. As a result, many patients with HIV-1 infection suffer from weight loss, various forms of undernutrition and, eventually, severe metabolic wasting. Indeed, many patients appear to die from the consequences of progressive wasting rather than from the viral infection itself.

Regarding studies in subjects with Acquired Immune Deficiency Syndrome (AIDS), there is limited evidence to indicate an association between lipid alteration and disease manifestations of this syndrome. Patients with AIDS exhibit lower total plasma lipid levels and lower levels of individual fatty acids along with higher proportions of the metabolites of arachidonic acid (22:4ω6 and 22:5ω6) (2). While dramatic nutritional consequences are most apparent in the later stages of HIV-1 infection, relatively little is known regarding the nutritional changes that occur during earlier stages of HIV-1 infection. Our research group has recently documented multiple nutritional abnormalities, in association with immune dysfunction, in early HIV-1 infection (3–5). Investigation of the nutritional aspects of HIV-1 infection has been oriented largely toward the clinically advanced stages of HIV-1 infection (that is, AIDS). Clinical nutrition intervention strategies oriented toward earlier stages of the disease might be utilized to delay disease progression. Moreover, they may allow for optimal use of anti-viral chemotherapy, such as zidovudine, dideoxycytidine and soluble CD4.

In this report, a comparison of total plasma fatty acid profiles between HIV-1 seronegative and HIV-1 seropositive asymptomatic homosexual men is documented in order to establish whether changes in plasma fatty acid profiles occur in asymptomatic HIV-1 infection and, if so, whether any of these changes are associated with immune processes.

MATERIALS AND METHODS

Subjects. Subjects recruited for the present investigation were 36 male homosexuals aged a mean of 30.8 years (range 23–45 yr) who were documented to be HIV-1 seropositive (confirmed by Western Blot technique), but who were asymptomatic other than for persistent generalized lymphadenopathy (CDC Stage III) (6,7).

No subject admitted to other risk factors for HIV-1 infection (for example, intravenous drug abuse). All 36 subjects were free of significant past medical history that might influence nutritional status (for example, diabetes mellitus, inflammatory bowel disease) and were not taking any medications known to interfere with nutritional status (for example, isoniazid or cycloserine). Moreover, no subject was or had been involved in any anti-viral chemotherapy protocol (for example, zidovudine, ribavirin, interferon).

The control subjects (n = 17) in the first population were 17 homosexual males aged a mean of 37.7 yr (range 25–64 yr) and were documented to be HIV-1 seronegative. Other than the HIV-1 seronegativity and lack of evidence for persistent generalized lymphadenopathy, this group of controls was matched to the HIV-1 seropositive study subjects. They were of similar age, socio-economic and

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Abbreviations: AIDS, Acquired Immune Deficiency Syndrome; ARC, AIDS-related complex; cpm, counts per minute; FAME, fatty acid methyl esters; HIV-1, Human Immunodeficiency Virus 1; NK, natural killer; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PUFA, polyunsaturated fatty acids; PWM, pokeweed mitogen.

ethnic characteristics, and had a similar aggregate level of education.

Dietary history. All subjects were questioned with respect to their dietary intake by means of Willett's semiquantitative food frequency questionnaire, currently widely employed in epidemiological investigations examining nutritional variables (8).

Clinical examination. All subjects were administered a complete medical history and general physical examination. Subjects were examined for any evidence of nutritional deficiencies or excesses. The comprehensive results of these examinations have been reported elsewhere (5). Self-report measures were recorded for current and historical alcohol use, recreational use of illicit substances (marijuana, cocaine, amphetamines, amyl nitrates, etc.) and smoking (estimated pack use).

Biochemical determinations. Venous blood samples were obtained at 0800 hours for all subjects from the left antecubital vein after a 12-h fast. The schedule was adhered to rigidly, in order to minimize the influence of the last meal and diurnal biochemical variation. Blood was collected in heparinized tubes that were centrifuged immediately at 2000 × *g*. Both the buffy coat and plasma were removed after centrifugation. All plasma samples were stored at −70°C until ready for analysis.

Total plasma lipids were extracted utilizing the Bligh and Dyer method (9). The lipid extracts were dried by flash evaporator and reconstituted in 2 mL methanol and 1 mL benzene containing a 17:0 fatty acid standard and 0.2 mL acetylchloride as catalyst. The tubes were allowed to stand overnight to quantitatively convert all fatty acids to their methyl esters (FAME). After adding 5 mL chilled phosphate buffer (final pH 5.0), the benzene layer containing FAME rose to the top and was transferred to small conical test tubes, and dried in a stream of N_2 at 50°C. The residual FAMEs were reconstituted in 25 μL hexane and injected into 1-μL aliquots. FAME were separated and quantified by gas-liquid chromatography (GLC Model 5880 with a flame-ionization detector Hewlett-Packard, Palo Alto, CA), with a 17:0 fatty acid used as an internal standard. The temperature program used was 160-260°C, increasing at 4°C/min. The carrier gas was nitrogen with a flow rate of 20 mL/min. The peaks were resolved by use of a 2.5-m glass column packed with 10% ethylene glycol succinate on 100 supelcoport (Supelco Inc., Bellefonte, PA). Individual fatty acids were identified and quantified by comparison of retention time against known standards (Sigma Chemical Co., St. Louis, MO).

Immunological determinations. Heparinized blood samples were drawn for lymphocyte surface markers (CD4 and CD8), assays of lymphocyte blastogenesis and of natural killer (NK) cell cytotoxicity. Flow cytometry and monoclonal antibodies were employed to quantitate members of circulating T helper/inducer (CD4) and T suppressor/cytotoxic (CD8) cell number (10). These studies were performed using two-color direct immunofluorescence with monoclonal antibodies conjugated to phycoerythrin to measure the CD4 population or to fluorescein isothiocyanate to measure the CD8 cell population. Both monoclonal antibodies were obtained from Coulter Immunology (Miami, FL).

Lymphocyte blastogenesis in response to the mitogen phytohemagglutinin (PHA), a T cell mitogen, and pokeweed mitogen (PWM), a T cell dependent B cell mitogen, was assessed in triplicate 72-h cultures (11). Assessment of tritiated thymidine incorporation during an additional 6 h of incubation was used to determine the degree of lymphocyte transformation, such transformation serving as an *in vitro* correlate of *in vivo* functional immunocompetence. The appropriate dilution of each mitogen was dispensed in 100-μL aliquots in triplicate to wells of a U-bottom microtiter plate (Costar, Cambridge, MA). Each mitogen was tested at three levels as follows: PHA (Wellcome Diagnostics, Dartford, England) 0.5, 5 and 10 μg/mL; and PWM (GIBCO Laboratories, Chagrin Falls, OH) diluted 1:40, 1:20 and 1:10. Three wells per each sample row received only growth medium of RPMI 1640 containing 100 units penicillin and 50 units streptomycin and 1 mM L-glutamine (GIBCO, Grand Island, NY). Heparinized peripheral blood was diluted 1:5, or 1:10, 1:20, 1:40 and 1:80 in some experiments, with growth medium, and 100 μL of the diluted blood was dispensed to all wells. PHA cultures were incubated for 72 h, and PWM cultures were incubated for 168 h (±2 h) in a humidified atmosphere of 5% CO_2 at 37°C. After the appropriate incubation time, 25 μL of tritiated thymidine was added to each well (1 μCi per well) and cultures were returned to incubation for an additional 6 h (±2 h). At the end of incubation, cultures were placed in the refrigerator at 4°C and harvested after 2 d. Cultures were harvested (Titertek Cell Harvester, Flow Laboratories, McLean, VA) onto glass filter paper disks that were dried, immersed in scintillation cocktail and counted on a beta scintillation counter (LKB Instruments, Rockville, MD) to determine counts per minute (cpm) of incorporated tritiated thymidine. Results were expressed as mean of net cpm of triplicate cultures. The number of lymphocytes present in the cultures was determined from total leukocyte count times percent lymphocytes observed from differential blood smear analyses. Mean of net cpm was transformed to mean cpm/100,000 lymphocytes. Calculations were done by using an MBasic program and an Apple II Plus microcomputer.

NK cell function was evaluated by assays of cytotoxicity. NK cell activity was assessed on a true effector cell basis, thereby minimizing the influence of changes in cell number upon assays of cell function (12). The technique is based upon a ^{51}Cr-release assay, using a K562 tumor target cell line. The assay was performed utilizing whole blood, thereby creating an *in vitro* environment closer to that in which the cell must function *in vivo*, than would be possible employing fractionated mononuclear cells. NK effector cell number was measured using the monoclonal antibody NKH.1 and fluorocytometry.

One-color indirect immunofluorescence surface marker analysis was performed using NKH.1 (Coulter Immunology, Hialeah, FL). Aliquots of 150 μL well-mixed heparinized peripheral blood were incubated with the appropriate monoclonal antibody or isotype control for 15 min at 4°C with shaking. Erythrocytes were lysed with 3 mL lysing buffer (0.155 M NaCl, 0.01 M $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid) for 10 min. The residual cells were washed two times with phosphate-buffered saline (PBS) and incubated with fluorescein isothiocyanate-conjugated goat antimouse IgG antibody for 15 min at 4°C on ice. The stained cells were washed two times with RPMI 1640 (GIBCO) and resuspended in 1 mL of

PBS and analyzed on the flow cytometer as above. The percent positively stained cells was determined using the Immunohistogram subtract software (Coulter, Hialeah).

A whole blood ^{51}Chromium release assay was used to determine cell-mediated cytotoxicity (CYT) to the NK-sensitive erythroleukemic K562 cell line. Briefly, triplicate aliquots of 150 μL heparinized peripheral blood from each sample were dispensed into wells of 96-well flat-bottom tissue culture trays (Costar, Cambridge, MA). ^{51}Cr-labeled (New England Nuclear, Boston, MA) log-phase K562 target cells at several dilutions, including 2×20^6 cells/mL, 1×10^6 cells/mL, 0.5×10^6 cells/mL were incubated with the blood samples. After 4 h incubation in a humidified atmosphere of 5% CO_2 at 37°C, cell-free supernatants were removed and counted on a gamma counter (LKB Instruments). Natural CYT was expressed as % CYT:

$$\%CYT = \frac{(ER - b) \times \frac{Vt - (Vb \times H)}{Vt} - (SR - b)}{(TR - b) - (SR - b)} \times 100 \quad [1]$$

where ER is the mean cpm of experimental release of the specimen, SR is the mean cpm spontaneous release, TR is the mean cpm total release, Vt is the total volume in well, Vb is the volume of blood in well, H is the specimen hematocrit and b is instrument background. Calculations were done using an MBasic program on an Apple II Plus microcomputer. Percent spontaneous release was less than 20%.

Statistical methods. Comparisons of fatty acid profiles in the HIV-1 seropositive and seronegative groups were made using Wilcoxon rank sums test for independent samples. Most of the immunologic parameters had non-normal distributions. Simple associations between measures of plasma fatty acids and immune parameters (CD4 cell number, CD8 cell number, CD4/CD8 ratio, PHA and PWM response and NK cell cytotoxicity) were determined using Spearman rank order correlations with $P < 0.05$ considered statistically significant for all measurements. Base ten ($\log_{10}$) logarithmic transformations successfully normalized the distributions of these measures, and the $\log_{10}$ transformed values were used in all multivariate analyses. Transformed measures are noted wherever used.

RESULTS

The relative amount of each of the fatty acids measured, expressed as percent of total plasma fatty acids, is described in Table 1. Both palmitic (16:0) and stearic (18:0) acids were significantly higher in the HIV-1 seropositive group ($P = 0.002$). Arachidonic acid (20:4) was significantly lower in the HIV-1 seropositive group ($P = 0.001$).

Comparisons were also made of fatty acids with similar ω unsaturation structure (Table 2). Total saturated fatty acids (16:0 and 18:0) were significantly higher in the HIV-1 seropositive men ($P = 0.0001$). Total ω6 polyunsaturated fatty acids (18:2 and 20:4) were significantly lower in the HIV-1 seropositive group ($P = 0.0027$). There were no statistically significant differences in the amounts of monounsaturated (16:1 and 18:1) and ω3 polyunsaturated (18:3, 20:5 and 22:6) fatty acids between the two groups of subjects.

TABLE 1

Fatty Acid Profiles[a]

	HIV− (n = 17)	HIV+ (n = 36)
16:0	20.3 ± 1.86[b]	22.2 ± 1.96[b]
16:1ω7	2.7 ± 0.698	3.0 ± 0.99
18:0	6.6 ± 0.54[b]	7.4 ± 0.86[b]
18:1ω9	23.6 ± 2.31	24.4 ± 3.23
18:2ω6	35.5 ± 3.53	33.4 ± 4.55
18:3ω3	0.6 ± 0.34	0.6 ± 0.75
20:4ω6	7.7 ± 1.73[c]	5.9 ± 1.89[c]
20:5ω3	0.4 ± 0.24	0.6 ± 0.57
22:6ω3	1.9 ± 1.50	1.6 ± 4.74

[a]Values are expressed as mean % total plasma fatty acids ± SD. Comparisons were made between HIV− subjects and HIV+ subjects using the Wilcoxon Rank Sum test.
[b]$P = 0.001$.
[c]$P = 0.0008$.

The ratios of the elongation and desaturation products of the essential fatty acids were compared (Table 3). The ratio of linoleic to arachidonic acid was significantly higher in the HIV-1 seropositive group ($P = 0.0213$). There were no differences in the ratios of either linolenic to eicosapentaenoic acids, nor in eicosapentaenoic to docosahexaenoic acids.

Both groups consumed a similar amount of calories (3297 kcal ± 1399.6 in the seropositive *vs.* 2549 kcal ± 1144.9 in the seronegative) as well as total fat (65.9 g ± 43.41 *vs.* 38.7 g ± 17.64) and polyunsaturated fats (6.3 en% ± 1.80 *vs.* 6.1 en% ± 2.32). The percentage polyunsaturated fat in the diet correlated with the 18:2 to 20:4 ratio in seronegative subjects ($r = 0.6526$; $P = 0.0114$). It also was inversely related to plasma total saturated fatty acids in seropositive subjects ($r = -0.3326$; $P = 0.0443$). No significant associations between plasma levels of total ω6 polyunsaturated fatty acids and caloric intake, or total fat consumed, were observed in either HIV-1 seronegative or seropositive subjects. Alcohol intake was also compared between seropositive and seronegative subjects. There was no statistically significant difference between the two groups ($P = 0.8337$).

Immune parameters were determined in HIV-1 seronegative and HIV-1 seropositive individuals. CD4 cell count correlated both with total plasma saturated fatty

TABLE 2

Comparisons by Degree of Unsaturation of Fatty Acids[a]

	HIV− (n = 17)	HIV+ (n = 36)
SFA	27.0 ± 2.00[b]	29.6 ± 2.13[b]
MUFA	26.3 ± 2.80	27.4 ± 4.00
ω6 PUFA	43.2 ± 4.23[c]	39.2 ± 4.95[c]
ω3 PUFA	2.9 ± 1.66	2.8 ± 1.54

[a]Values are expressed as mean % of total plasma fatty acids ± SD. Comparisons were made between HIV− subjects and HIV+ subjects using the Wilcoxon Rank Sum test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
[b]$P = 0.0001$.
[c]$P = 0.004$.

TABLE 3

Comparisons of Ratios of Fatty Acids[a]

HIV−	HIV+	
18:2 to 20:4	4.86 ± 1.374[b] n = 17	6.72 ± 4.745[b] n = 35
18:3 to 20:5	1.66 ± 1.463 n = 15	0.90 ± 0.570 n = 27
20:5 to 22:6	0.28 ± 0.223 n = 17	0.58 ± 0.807 n = 32

[a]Values are expressed as mean ratios of % of total plasma fatty acids ± SD. Comparisons were made between HIV− subjects and HIV+ subjects using the Wilcoxon Rank Sum test.
[b]$P = 0.0213$.

acids ($r = 0.6593$; $P = 0.0055$) and with the ratio of 18:2 and to 20:4 ($r = 0.5235$; $P = 0.0374$) only in seronegative subjects. In HIV-1 seropositive participants the mitogenic response to PHA was found to correlate with both total plasma ω6 polyunsaturated fatty acids ($r = -0.36$; $P = 0.027$) and with the ratio of 18:2 to 20:4 fatty acids ($r = 0.33$; $P = 0.046$).

DISCUSSION

The present findings indicate that the polyunsaturated fatty acid profile is different from controls during the early stages of HIV-1 infection. The mean total amount of ω6 polyunsaturated fatty acids (18:2 and 20:4) was lower in the HIV-1 seropositive subjects compared to HIV-1 seronegative subjects. This was also reflected in higher total saturated fatty acids (16:0 and 18:0) in HIV+ subjects. The mean ratio of linoleic to arachidonic acid (18:2 to 20:4) was higher in the HIV+ group, but there were no statistically significant differences between HIV− and HIV+ subjects when total monounsaturated (16:1 and 18:1) and total ω3 polyunsaturated (18:3, 20:5, and 22:6) fatty acids were compared.

Consistent with the present findings, alterations in plasma fatty acid profiles have been found in the later stages of HIV-1 infection. Begin *et al.* (13) collected blood samples from 11 patients with fully developed AIDS, and lipid fractions were analyzed. Total lipid levels were approximately 75% of normal, and the absolute plasma levels of stearic, oleic and linoleic acids were significantly lower, although proportions of arachidonic acid were not different between the two groups. In another study, Christeff *et al.* (14) found an increase in the relative percentage of polyunsaturated fatty acids in the free fatty acids from plasma of AIDS-related complex (ARC) and AIDS patients. However, with more advanced AIDS disease, the differences in polyunsaturated fatty acids were lost. Differences in results from our study may be attributed to the stage of the disease. Although not clearly specified, it is apparent that the subjects in studies performed by others were CDC Stage IV, in a more advanced state of disease and malnutrition than our subjects, who, except for lymphadenopathy, were asymptomatic.

Changes in lymphocyte membrane phospholipids also occur with progression of HIV-I disease. Klein *et al.* (15) noted an increase in CD4 membrane phospholipid linoleic acid in asymptomatic seropositive homosexuals as the CD4 count dropped below 500×10^6/L. In contrast, symptomatic subjects (ARC or AIDS) showed an increase in oleic acid, and a decline in linoleic and arachidonic acids. These authors suggest that the decrease in concentrations of ω6 fatty acids may represent activation of cyclooxygenase pathways (15).

There is some evidence that serum fatty acid profiles may be altered by other viral infections. Williams *et al.* (16) took monthly measurements of serum total fatty acid profiles of twenty otherwise healthy college students following acute Epstein-Barr virus infection. Clinical symptomatology, such as increased physical malaise, coincided with changes in fatty acid profiles. Specifically, lower levels of arachidonic acid and a reversal of the usual ratio of linoleic and oleic acids were noted. Low levels of linoleic acid content were observed in a subset of subjects who showed continued clinical symptoms. The authors estimated that fatty acid elongation enzyme function was normal, but that desaturation enzyme activities were lower than normal.

The HIV-associated changes in fatty acid profiles may have correlates in other disease states. Alden *et al.* (17) studied the plasma phospholipid polyunsaturated fatty acid profiles in 22 critically ill, hypermetabolic patients. Compared to controls, all patients showed low levels of linoleic and arachidonic acids. However, because levels of docosapentaenoic acid (22:5 ω6), a metabolite of arachidonic acid, were increased, total ω6 metabolites were not different from controls. This study suggests that elongation and desaturation of arachidonic acid in sepsis may be accelerated.

Other inflammatory processes may be associated with altered polyunsaturated fatty acid utilization. Melnick and Plewig (18) have hypothesized that inflammatory skin diseases, such as atopic dermatitis, are linked to alterations in ω6 fatty acid metabolism. They have suggested that reduced formation of Δ6 desaturate products (specifically dihomo-γ-linolenic acid) results in diminished T cell maturation. The levels of linolenic, dihomo-γ-linolenic and arachidonic acids are decreased in patients with atopic dermatitis, whereas the levels of linoleic acid are higher than normal (19,20).

Diseases of the liver also may alter plasma total fatty acid profiles. Almost universally, several groups of investigators have noted a general decrease in polyunsaturated fatty acids in cirrhotic liver disease (21–25). This pattern appears to be exacerbated by alcohol abuse (21) or by malnutrition (22). Biagi *et al.* (26) have measured Δ6 desaturase activity in microsomes from liver specimens of normal and cirrhotic subjects, and have observed a significant decrease in Δ6 desaturase activity.

The changes in fatty acids were also correlated with immune function in our study. The response to PHA in HIV+ subjects correlated inversely with total plasma ω6 fatty acids, and directly with the 18:2 to 20:4 ratio, suggesting that reduced production of arachidonic acid is associated with preservation of the normal lymphocyte response to PHA.

It is known that essential fatty acids have potent effects on immune function. In general, linoleic and arachidonic acids, when added *in vitro*, suppress lectin-induced lymphocyte transformation (27). These effects may be produced by changes in eicosanoic production. In many cases PGE_2 (of which the precursor is linoleic acid) suppresses T cell function, such as response to mitogens

and antigens, rosette formation, generation of cytotoxic cells and production of lymphokines.

We conclude that: (i) early HIV-1 infection is associated with lower plasma ω6 PUFA than controls; (ii) the proportion of arachidonic acid in early HIV-1 infection is lower; (iii) these observations are not due to differences in dietary intake and (iv) the changes in the plasma fatty acid profile correlate with some indices of immune function. Infection with HIV-1 may be associated with alterations of the hepatic enzymes necessary for desaturation and elongation of linoleic acid, which may be reflected in modulation of immune function *via* prostaglandin synthesis.

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Atherosclerosis and Plasma and Liver Lipids in Nine Inbred Strains of Mice

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Nine inbred strains of mice, which are progenitors of recombinant inbred sets, were evaluated for aortic lesion formation and plasma and liver lipid levels. This survey was done to determine if a semi-synthetic high-fat diet could elicit the same extent of diet-induced atherosclerosis as that observed in mice fed a natural ingredient high-fat diet and to discover strain-specific plasma and liver lipid variants for future genetic characterization. Evaluation of aortic lesions after 18 wk of diet consumption showed that strains C57BL/6J, C57L/J, SWR/J and SM/J were susceptible to atherosclerosis and that A/J, AKR/J, C3H/HeJ, DBA/2J and SJL/J were relatively resistant. High-density lipoprotein cholesterol (HDL-C) levels were negatively correlated to lesion formation. Susceptible strains had decreased HDL-C levels when switched from chow to the semi-synthetic high-fat, high cholesterol diet, whereas resistant strains either showed no change or a slight increase in HDL-C levels. The exception to this pattern was found in SM mice, which were susceptible to aortic lesion formation but maintained the same HDL-C level on both chow and high-fat diets. HDL size differed among the strains, and levels of plasma apolipoprotein A-I and A-II correlated with HDL-C levels. Liver damage was not correlated to HDL-C levels or to susceptibility to atherosclerosis. Mice from strain A, which are resistant to atherosclerosis, had evidence of liver damage as observed by elevated levels of plasma alanine aminotransferase activity, by liver histology, by increased liver weight and by exceptionally high hepatic cholesterol content. For all strains, the levels of liver cholesterol and triglycerides were inversely correlated with each other; phospholipids did not vary greatly among strains. No remarkable differences in hepatic fatty acid profile were noted among the strains fed the atherogenic diet, but the fatty acid profile did differ considerably from that found in the diet itself.

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The mouse model is proving to be an invaluable tool for identifying genetic factors that affect heart disease and lipid metabolism because of the extensive genetic characterization of the inbred mouse strains (1), the special tools available, such as congenic, transgenic and recombinant inbred strains, the reproducible method for measuring aortic lesions (2) and the adaptation of methods to quantitate lipids in small blood volumes available from a single mouse. Previously, inbred strains of mice have been shown to differ in susceptibility to atherosclerosis when fed a natural grain diet high in saturated fat and cholesterol. The diet which was used in these studies was not chemically defined, and measurements of combined plasma very low density lipoprotein (VLDL) and low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) which have been shown to be associated with heart disease risk in humans were not done (3-5).

In this report, we sought to further develop the mouse model for the study of heart disease and lipoprotein metabolism. The semi-synthetic atherogenic diet which was designed to produce fatty streak lesions but minimize pathological changes in liver and gallstone formation in strain C57BL/6 is utilized in this study (6). However, cocoa butter was replaced by dairy butter as the fat source. The first question we asked was whether susceptibility to atherosclerosis would remain the same in nine inbred strains of mice fed a semi-synthetic high-fat and cholesterol diet rather than the natural ingredient diet used previously. Four susceptible strains (C57BL/6, C57/L, SWR and SM) and five resistant strains (A, DBA/2, AKR, C3H/He and SJL) were chosen for this study. These inbred strains of mice are progenitors of recombinant inbred sets. Secondly, we measured baseline differences in plasma lipid parameters and subsequent changes in these parameters in response to high-fat feeding in order to identify genetic variants in progenitors that could be further characterized and eventually mapped using recombinant inbred strains. In addition, because the liver is intimately involved in lipoprotein metabolism, the composition and levels of liver lipids were determined. The possible associations of the plasma and liver lipids with atherosclerosis susceptibility were also examined.

MATERIALS AND METHODS

Chemicals and diet. Cholesterol oxidase, bovine pancreas cholesteryl ester hydrolase, peroxidase and the plasma alanine aminotransferase assay kit were from Sigma Chemical Co. (St. Louis, MO); the cholesterol reagent kit No. 23691 was from Boehringer Mannheim (Indianapolis, IN); the triglcyeride reagent set No. 46676 was from Seradyn Inc. (Indianapolis, IN); triglyceride blank blend No. 10021 was from Craig Bioproducts (Streamwood, IL); chemicals for gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA); gradient gels (4-30% polyacrylamide) were from Pharmacia (Piscataway, NJ); Oil red O was from Aldrich Chemical Company (Milwaukee, WI); hematoxylin and light green were from Fisher Scientific Co. (Dallas, TX); gentamycin sulfate was from U.S. Biochemical Corp. (Cleveland, OH); and fatty acid methyl ester mixtures were from Supelco (Bellefonte, PA). Other chemicals utilized were of reagent grade or better. Components for the purified diets were purchased from ICN

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Abbreviations: ALT, alanine aminotransferase; apo A-I, apolipoprotein A-I; apo-II, apolipoprotein A-II; BW, body weight; EDTA, ethylenediaminetetraacetic acid; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; VLDL, very low density lipoprotein.

Biochemicals, Inc. (Cosa Mesa, CA) with the exception of Mazola corn oil (Best Foods, Englewood Cliffs, NJ). Laboratory rat chow (No. 5012) containing 4% fat was obtained from Ralston Purina (St. Louis, MO).

Preparation of diets and formation of the diet mix into pellets has been described previously (6). The high saturated fat diet contained 15% dairy fat, 50% sucrose, 20% casein, 1% corn oil, 5.07% cellulose, 5% AIN-76 mineral mix, 1% AIN-76 vitamin mix, 1% choline chloride, 0.3% DL-methionine, 0.13% DL-α-tocopherol, 0.5% sodium cholate and 1% cholesterol. Of the 1% cholesterol contained in the diet, 0.03% came from the dairy butter. In order to obtain 15% dairy butter, 18.45 g butter was used/100 g diet because of the 18.9% water content of butter.

Animals. Female C57BL/6J (C57BL/6), C57/LJ (C57/L), SWR/J (SWR), SM/J (SM), A/J (A), AKR/J (AKR), C3H/HeJ (C3H), DBA/2J (DBA/2) and SJL/J (SJL) mice, 4–8-wk-old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a room illuminated from 7 a.m. through 7 p.m. All animals were allowed to adapt to the environment for at least two weeks prior to dietary treatment and were provided free access to food and water throughout the experiment. Weight gain was monitored every three weeks, and food intake was monitored at weeks 3, 7 and 14. Six female mice were used from each strain for plasma lipid analysis and three were used for liver lipid analysis. After 18 wk of diet consumption, 0 of 6 mice from strains DBA/2, C57BL/6, SWR, C57L and A, 1 of 6 mice from strains SM, SJL and C3H and 3 of 6 mice from strain AKR had died. The reason for death in strains SM, SJL and C3H is unknown. The AKR mice died from exposure due to a water bottle malfunction.

Collection of blood and tissues. Prior to any blood or tissue collection, mice were fasted by removing their food at 5 p.m. on the day prior to the experiment. Blood was obtained from the tail vein of the mice after the two-week adaptation period for baseline measurements, and again after four weeks of consuming the semi-synthetic high-fat and cholesterol diet. At the termination of the experiment, blood was obtained from the brachial artery. Blood was mixed in chilled tubes with ethylenediaminetetraacetic acid (EDTA), sodium azide and gentamycin sulfate at final concentrations of 2 mM, 0.05 mg/mL and 0.05 mg/mL, respectively. Plasma was obtained by centrifugation of whole blood for 5 min at 12,000 $\times$ *g* at 4°C.

Upon termination of the experiments, the heart and the upper section of the aorta were removed and placed in 0.9% saline at room temperature. After one hour, the heart was trimmed of extraneous tissue and placed in 10% saline buffered formalin for 24 h at room temperature. Livers were blotted, quickly frozen in liquid nitrogen and stored at −70°C for lipid analysis. A portion of the liver was placed in a Bouin's fixative, embedded in paraffin wax, sectioned and stained with hematoxylin and eosin. Necropsy observations concerning the stomach and intestines were recorded.

Plasma lipids, lipoproteins and apolipoproteins. Plasma total cholesterol (TC), free glycerol and total glycerol were determined by commercial colorimetric enzymatic assay. HDL-C was measured after selective precipitation of LDL and VLDL with polyethylene glycol (7). The combined VLDL and LDL-C was calculated as the difference between TC and HDL-C. Plasma triglyceride concentrations were estimated by subtraction of free glycerol from total glycerol. Lipid measurements are given as mg/dL ± SEM. Plasma concentrations of apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II) were estimated by radial immunodiffusion (8). Purified mouse apo A-I and apo A-II served as standards.

Total plasma lipoproteins ($d < 1.21$) were obtained by density ultracentrifugation of pooled plasma from five mice of each strain (9) and subjected to nondenaturing gradient gel electrophoresis (10). Gels were stained with Coomassie blue G-250 to visualize HDL.

Liver lipids and plasma alanine aminotransferase (ALT) activity. Lipids, extracted from livers by the method of Folch *et al.* (11), were assayed for triglyceride (12), cholesterol (13) and phosphorus (14). Phosphorus values were multiplied by a factor of 25 to estimate liver phospholipid content, assuming that phospholipids contain approximately 4% phosphorus by weight based on an average molecular weight for phospholipid of 775. Plasma ALT activity was measured using the Sigma Kit No. DG591K which is based upon a coupled reaction in which nicotinamide adenine dinucleotide is oxidized.

The fatty acyl group composition of lipid extracts from liver was determined by profiling the long-chain fatty acyl groups after transesterification of the fatty acids. Gas-liquid chromatography was performed on a Shimadzu 95A Gas Chromatograph (Columbia, MD) equipped with a 30 m $\times$ 0.25 mm fused silica SP 2330 column (Supelco) and a flame-ionization detector. Standard fatty acid methyl ester mixtures were used to identify the fatty acid methyl esters in the samples.

Evaluation of atherosclerotic lesions. Evaluation of aortic lesions has been described in detail by Paigen *et al.* (2). Briefly, mouse hearts were fixed, stored in 4% phosphate buffered formaldehyde and embedded in 25% gelatin. After removing the lower two-thirds of the heart, the remaining tissue was sectioned on a cryostat at −25°C. Alternate 10 μm sections were saved on slides. Sections were stained with aqueous Oil Red O for neutral lipid and hematoxylin for nuclei and basophilic tissue and counterstained with light green. Five sections at 80 μm intervals were evaluated for the cross sectional area of lesions beginning where the aorta was rounded and valves appeared distinctly through to the endpoint where the valves disappeared, a distance of approximately 350 μm.

Statistical analysis. The Number Cruncher Statistical System, Version 4.21 1/86 (Kaysville, UT) and Statview, Version 1.0 6/85 (Calabasas, CA) was used for statistical analysis. Comparison of data from more than two groups was analyzed by using one-way analysis of variance with Fisher's least significant difference test to determine which means were significantly different at $P < 0.01$. Unpaired *t*-test was used when only two groups were compared. Correlation between aortic lesions and lipid parameters measured were tested by linear regression analysis.

RESULTS

Atherosclerosis susceptibility. The susceptibility to lesion formation is similar in the nine inbred strains of mice fed the semi-synthetic high-dairy-fat diet to that reported earlier (5), when these same strains were fed a natural ingredient diet with cocoa butter as the fat source (Fig. 1).

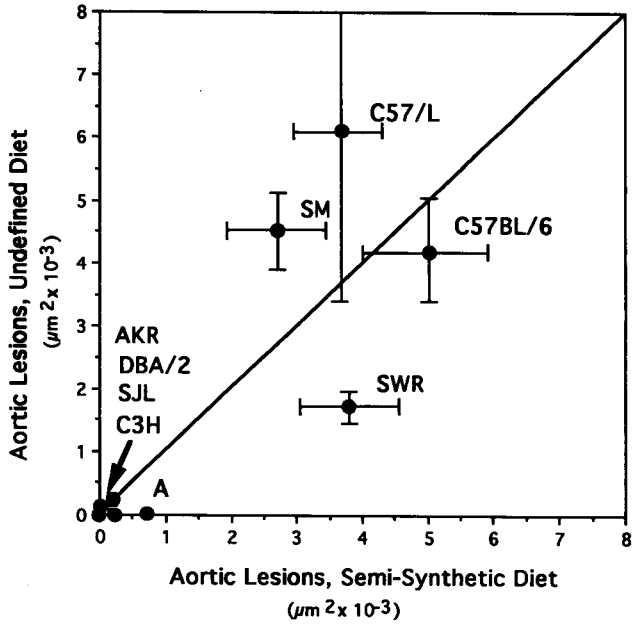

FIG. 1. Comparison of aortic lesions in nine inbred strains fed two different atherogenic diets. The aortic lesion sizes are expressed as the mean size per cross section in $\mu m^2 \times 10^3$. Lesion data for the natural ingredient diet are from Reference 5.

C57BL/6, C57L, SWR and SM are susceptible to lesion formation while the remaining five strains, namely A, AKR, C3H, DBA/2 and SJL, are relatively resistant to atherosclerosis. This difference among strains could not be accounted for by food consumption and weight gain (Table 1) as these parameters were not significantly different from that expected based on the relative sizes of these inbred strains maintained at The Jackson Laboratory. Therefore, the reduction in dietary cholesterol from 1.25% in the undefined diet to 1% in the semi-synthetic diet and the change in the source of fat from cocoa to dairy butter did not affect susceptibility to aortic lesion formation. The intra-strain variability and extent of lesion area was also similar between the two experiments. The one exception was strain SWR, where the lesion area was larger in mice consuming the semi-synthetic rather than the undefined diet (3804 ± 741 *vs.* 1690 ± 280 μm^2, respectively).

TABLE 1

Initial Body Weight, Body Weight Gained and Food Intake of Mice Fed a High-Saturated Fat Diet[a]

Strains	Body weight (g)			Food intake[b] g/d/20 g BW
	Initial	Final	% Normal	
C57BL/6	19.0 ± 0.5	22.4 ± 0.6	100	1.8
C57L	17.4 ± 0.6	24.2 ± 0.9	99	2.7
SWR	18.6 ± 0.6	23.9 ± 0.4	n.a.[c]	2.2
SM	13.8 ± 0.8	20.4 ± 1.0	100	2.7
A	18.0 ± 1.1	21.0 ± 1.0	75[d]	2.3
DBA/2	20.8 ± 0.4	28.7 ± 0.7	104	2.0
AKR	26.9 ± 1.2	44.3 ± 1.6	138	2.2
C3H	21.2 ± 0.8	32.9 ± 1.1	102	2.0
SJL	20.7 ± 0.8	24.9 ± 1.3	92	2.4

[a]Values represent the mean ± SE of at least five animals per group at 4 wk of high-fat diet consumption and at least three animals per group at 18 wk.
[b]Food intake was estimated by weighing food disappearance over a 3- to 5-d period; the values represent the mean of food consumed per 20 g body weight (BW) of mouse per day during weeks 3 through 4 of the experiment.
[c]n.a. = Not available.
[d]This strain lost weight during the last six weeks of the study, presumably because their livers were damaged.

Baseline plasma lipid levels in the nine inbred strains of mice. Significant variation in basal plasma lipids (combined triglyceride and cholesterol) was noted among the strains studied (Fig. 2). Plasma triglyceride levels ranged from 26 ± 3 mg/dL in strain C57/L to 68 ± 5 mg/dL in strain SWR. Plasma cholesterol levels ranged from 45 ± 4 mg/dL in C57/L to 106 ± 4 mg/dL in C3H mice (Fig. 2B). As observed by others (15), the majority of cholesterol in mouse plasma, 71 to 87%, is found in the HDL fraction (Fig. 2C) with 13 to 29% in the VLDL + LDL fraction (Fig. 2D).

Plasma lipid levels in mice fed a diet high in fat and cholesterol for four weeks. Fasting total triglyceride levels did not reflect the high-fat content of the diet (*i.e.* 15% w/w) fed to the mice (Fig. 2A). Triglyceride levels either did not change from basal levels in strains C57BL/6, C57L, SWR, A, AKR, C3H and SJL, or significantly decreased by 38% in SM and 39% in DBA/2.

Unlike plasma triglyceride, total plasma cholesterol levels increased significantly in all strains fed the semi-synthetic atherogenic diet (Fig. 2B). The level of increase ranged from 131 to 295% over baseline values. The majority of the increase in plasma cholesterol could be found in the VLDL + LDL fraction (Fig. 2D). VLDL + LDL-C levels of the nine inbred strains clustered roughly into two groups with C57BL/6, SM, DBA/2, AKR and SJL having levels from 47 ± 4 to 67 ± 19 mg/dL and C57/L, SWR, A and C3H having levels from 94 ± 10 to 132 ± 10 mg/dL (Fig. 2D). HDL-C either decreased significantly from baseline values in strains C57BL/6, C57/L and SWR or showed no significant change in the remaining strains. The decrease in SWR, C57BL/6 and C57/L mice was 19, 32 and 37%, respectively (Fig. 2C).

Liver lipid levels in mice fed the atherogenic diet for 18 wk. Liver weights varied from 1.2 to 3.9 g/animal. When liver weight was normalized (g/100 g body weight) to account for differences in body size, strain A was exceptional in that its liver was more than twice the size observed in other strains. On gross examination, the livers of the animals from all strains fed the semi-synthetic high-fat diet tended to be paler than normal compared to animals fed laboratory chow. C57L, DBA/2 and SJL had livers that were closest to normal in color. This group was followed by strains C57BL/6, C3H, SM, SWR and AKR, which had paler-colored livers. The livers of strain A were chalky white and rigid. In general, the strains with the lowest levels of hepatic cholesterol were most normal in appearance, and as the hepatic cholesterol content of the liver increased, the livers became paler in color. Histological examination of livers (Fig. 3) were consistent with this interpretation. Lipid accumulation observed in the liver as clear areas in the cytosol was least in C57/L (Fig. 3, Panel B), intermediate in C57BL/6 (Fig. 3, Panel C), and most in strain A (Fig. 3, Panel D). As a gross indicator of liver damage, plasma ALT activity was measured. The

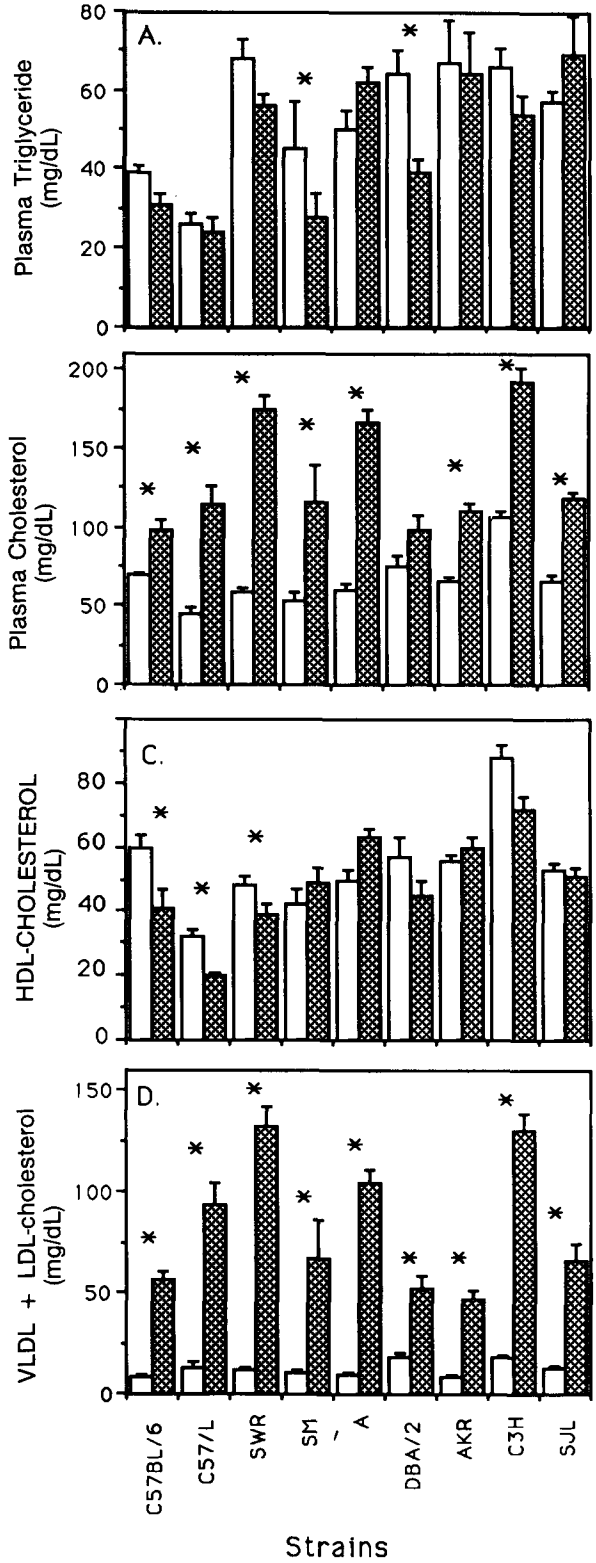

FIG. 2. Comparison of basal and high-fat levels of plasma triglycerides (A), cholesterol (B), high-density lipoprotein (HDL)-cholesterol (C) and combined very low density lipoprotein (VLDL) and low-density lipoprotein (LDL)-cholesterol (D) in nine inbred strains. The solid bars represent mean lipid and lipoprotein levels in mg/dL ± SE in the basal state, and the gray bars represent mean lipid and lipoprotein levels in mg/dL ± SE for animals fed the semi-synthetic atherogenic diet for four weeks. Significance ($P < 0.05$) between basal and high-fat values is indicated by a star above the bars.

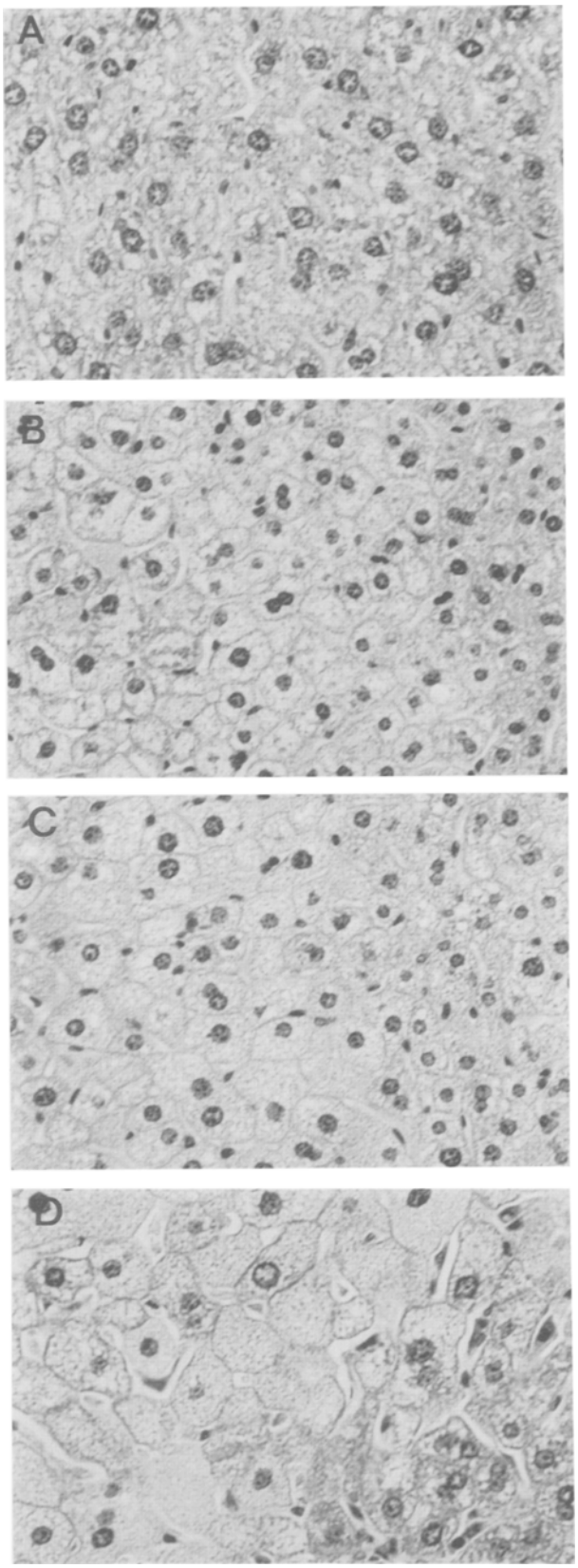

FIG. 3. Liver sections from mice fed chow (A) or the semi-synthetic diet for 7 wk (B–D) stained with hemotoxylin and eosin, 368×. Strain A (A), C57L (B), C57BL/6 (C), and Strain A (D).

TABLE 2

Comparison of Liver Weights and Plasma Alanine Aminotransferase (ALT) Activity in Nine Strains of Mice Fed a High-Saturated Fat Diet (for 18 wk)[a]

Strains	Liver weight		ALT activity[b]
	g/animal	g/100 g BW	μmol/min/mL
C57BL/6	1.5 ± 0.1[c]	6.8 ± 0.4[c,d]	55 ± 12[c,d]
C57L	1.4 ± 0.1[c]	5.9 ± 0.1[c]	29 ± 2[c]
SWR	2.1 ± 0.1[d,e]	8.9 ± 0.3[d]	105 ± 24[d]
SM	1.2 ± 0.1[c]	5.8 ± 0.8[c]	45 ± 16[c,d]
A	3.9 ± 0.1[f]	18.6 ± 0.9[e]	479 ± 28[e]
DBA/2	1.3 ± 0.1[c]	4.4 ± 0.2[c]	67 ± 19[c,d]
AKR	2.6 ± 0.1[e]	5.8 ± 0.2[c]	32 ± 11[c]
C3H	2.1 ± 0.1[d]	6.3 ± 0.2[c,d]	55 ± 8[c,d]
SJL	2.0 ± 0.2[d]	8.2 ± 0.4[d]	68 ± 15[c,d]

[a]Values represent the mean ± SE of at least three animals per group fed a high-saturated fat diet for 18 wk. Values in columns without common superscripts are significantly different, $P < 0.01$. [b]Animals that are fed chow or a low-fat synthetic diet have plasma ALT levels of 16.6 ± 3.1 or 30 ± 4 μmol/min/mL, respectively (Nishina, P.M., unpublished data).

two strains with the highest liver weight/100 g body weight, A and SWR, also had the highest ALT activity (479 and 105 μmol/min/mL, respectively), and both of these strains also had accumulated an extensive number of gallstones. Except for these two strains, plasma ALT levels were not remarkably elevated compared to ALT levels (17 ± 2 μmol/min/mL) measured in chow-fed C57BL/6 mice (Table 2).

Triglyceride, cholesterol and phospholipids were measured in order to determine what lipids were accumulating in the livers (Table 3). Liver cholesterol in C57BL/6 animals fed a low-fat diet is 5 ± 0.2 mg/g liver (6). The liver cholesterol of strains fed the high-fat, high-cholesterol diet ranged from 20 to 63 mg/g liver with strains DBA/2 and C57L accumulating the least, and strains C57BL/6 and A having the most cholesterol. The distribution of cholesterol into free and esterified cholesterol was examined; no significant differences among the strains were observed, and most cholesterol was of the esterified form with only 9 to 13% as free cholesterol (data not shown). Liver triglyceride levels ranged from 3 ± 1 to 24 ± 1 mg/g liver among the different strains. Strain A, which had the highest liver cholesterol concentration, had the lowest concentration of liver triglycerides. The accumulation of liver cholesterol in strain A mice fed a high-fat and high-cholesterol diet has been noted previously (16). Phospholipids, which are necessary for assembly of lipoproteins for secretion from the liver, were less variable than other lipids among strains. Correlation analysis of liver phospholipids, triglyceride or cholesterol showed that concentrations of phospholipids and cholesterol were negatively correlated ($r = -0.90$, $P < 0.01$) among the strains. This suggests that cholesterol may accumulate in livers which have lower concentrations of phospholipid. No correlation was found between plasma lipoproteins and any of the measured hepatic lipid concentrations after 18 wk of receiving the high-fat diet.

TABLE 3

Comparison of Liver Cholesterol, Triglyceride and Phospholipid (mg/g liver) in Nine Strains of Mice Fed a High-Saturated Fat Diet (for 18 wk)[a]

Strains	Cholesterol		Triglyceride	Phospholipid
	Total	Esterified/ free ratio		
C57BL/6	50 ± 4[c]	11 ± 0.5	14 ± 1.4[c,d]	20 ± 0.8[b]
C57L	29 ± 2[b]	7.7 ± 0.5	10 ± 1.9[b,c]	26 ± 1.2[c]
SWR	39 ± 2[c]	8.8 ± 0.6	5.8 ± 0.9[b]	22 ± 0.4[b]
SM	45 ± 6[c]	9.2 ± 0.4	24 ± 1.1[d]	24 ± 2.1[b,c]
A	63 ± 2[d]	7.7 ± 0.5	3.3 ± 0.8[b]	19 ± 0.4[b]
DBA/2	20 ± 1[b]	6.5 ± 0.2	20 ± 1.8[d]	26 ± 0.8[c]
AKR	40 ± 2[c]	8.8 ± 1.0	4 ± 1.7[d]	23 ± 0.3[b,c]
C3H	42 ± 2[c]	9.8 ± 0.3	13 ± 0.6[c]	22 ± 0.4[b,c]
SJL	39 ± 2[c]	8.9 ± 0.4	16 ± 2.6[c,d]	24 ± 0.9[b,c]

[a]Values represent the mean ± SE of at least three animals per group fed a high-saturated fat diet for 18 wk. Values in columns without common superscripts are significantly different, $P < 0.01$, as determined by Fisher's Least Significance Difference test.

In order to determine whether liver fatty acid profiles differed among the nine strains, livers from a minimum of three animals per strain were pooled and analyzed (data not shown[2]). The liver fatty acid profiles did not differ among the strains fed the high-fat diet; approximately 15 to 25% of the fatty acids were saturated, mainly in the form of palmitic acid, 50 to 80% were monounsaturated, mainly in the form of oleic acid, and 5 to 20% were polyunsaturated, mainly in the form of linoleic acid. However, the fatty acid profiles found in livers did differ considerably from those found in the diet where 73% of the fatty acids were saturated, 25% monounsaturated and 2% polyunsaturated.

Relationships between atherosclerosis susceptibility and plasma and liver lipids. Previous studies in mice have shown that the resistance to atherosclerosis cosegregates with elevated levels of HDL-C in crosses or in recombinant inbred strains derived from the susceptible strain C57BL/6 and the resistant strains C3H, BALB/c or A (17-19). In this survey, HDL-C levels in mice receiving the atherogenic diet were inversely related to aortic lesions size ($r^2 = -0.49$, $P < 0.05$). No other significant correlations were observed between aortic lesion formation and plasma or liver lipid levels.

Because of the importance of HDL-C levels in determining resistance to atherosclerosis, the possible biochemical differences in the HDL particles among the inbred strains were examined. The major apolipoproteins associated with these particles, A-I and A-II, were measured. The changes in HDL-C were mirrored by changes in apo A-I and A-II. The correlation among HDL-C, apo A-I and A-II was high; the correlation between HDL-C and apo A-I was 0.79 ($P < 0.0001$, Fig. 4B), between HDL-C and apo A-II was 0.70 ($P < 0.0001$, data not shown) and between apo A-I and apo A-II was 0.64 ($P < 0.0001$, Fig. 4A). Overall, the ratio between these apolipoproteins and HDL-C did not change significantly in inbred mice, whether fed a chow or a high-fat diet. Considerable variation in peak HDL particle sizes occurred among the mouse strains with no obvious correlation to atherosclerosis susceptibility or resistance (data not shown).

[2]A complete liver fatty acid profile of the nine strains of mice fed the semi-synthetic atherogenic diet will be supplied upon request.

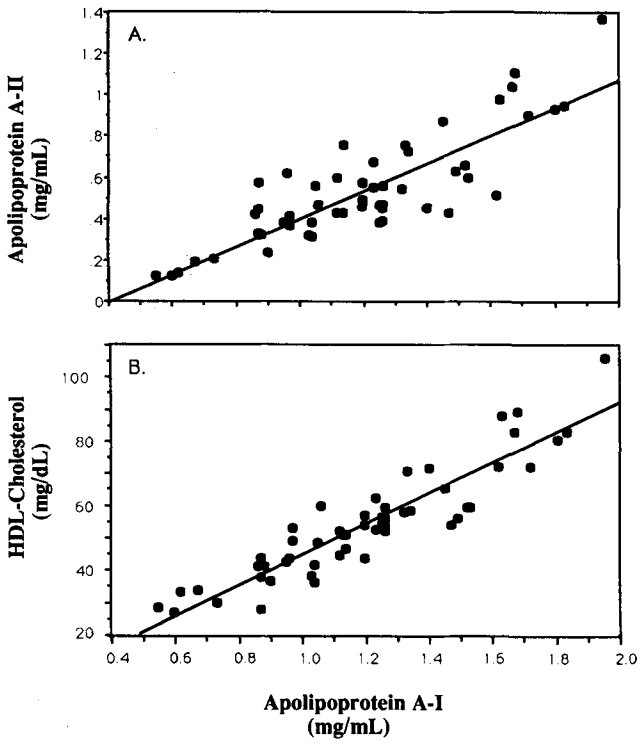

FIG. 4. Correlation analysis of apolipoproteins and high-density lipoprotein (HDL)-cholesterol in mice fed the semi-synthetic diet for four weeks. Panel A. Correlation of apolipoprotein A-II *vs.* apolipoprotein A-I. Panel B. Correlation of HDL-cholesterol *vs.* apolipoprotein A-I.

DISCUSSION

Atherosclerosis susceptibility. The relative susceptibility of nine inbred strains of mice to formation of fatty streak lesions in the aorta did not change when substantial changes were made in the atherogenic diet. The diet which we previously had used contained natural ingredients such as oats and corn, 1.25% cholesterol and cocoa butter as the primary fat source; the diet used in this study was a semi-synthetic diet with 1% cholesterol and dairy butter as the major fat source (5,6). Both diets contain 0.5% sodium cholate (w/w). The fact that the relative susceptibility to lesions did not change with diet indicates that previous conclusions about the genes that affect susceptibility to atherosclerotic lesion formation are applicable to more than one diet. However, it is interesting to note that diet can have an effect on the severity of the disease. Aortic lesion area was twice as large in SWR mice fed the semi-synthetic diet as compared to the undefined atherogenic diet.

The changes in VLDL + LDL-cholesterol and HDL-cholesterol are less than previously reported for C57BL/6 animals fed the natural ingredient atherogenic diet (5). The most likely explanation is the 20% decrease in cholesterol content of the semi-synthetic diet. Consistent with the less dramatic changes in lipoproteins, the length of time which animals needed to be fed the semi-synthetic diet to produce lesions of comparable size as the natural ingredient diet was 18 rather than 14 wk. However, the semi-synthetic diet has considerable advantages over the previous diet because it is defined and dietary components thought to affect development of heart disease can be varied one at a time to determine specific effects. In addition, the semi-synthetic diet produces fewer pathological changes in the liver and a decrease in gallstone formation (6); these pathologies might be further reduced by decreasing the cholesterol and or sodium cholate content of the diet. However, such a reduction would most probably increase the experimental time required to produce aortic lesions.

Liver lipids in response to high-fat diet. Correlation analysis showed no indication of a relationship between liver damage, measured by plasma ALT activity, and a decrease in HDL-C levels or atherosclerosis susceptibility. The disassociation of these two processes is most clearly demonstrated in strain A which shows no drop in HDL-C levels and the greatest liver damage, and strain C57/L, which has the lowest HDL-C and little liver damage when switched from a low- to high-fat diet.

The liver damage observed in these strains is consistent with the histopathology and appears to be associated with the accumulation of cholesterol in the liver. Undoubtedly, the cholesterol accumulated in the liver, in large part, arises from diet consumption. However, *de novo* hepatic cholesterol synthesis may also play a role in Strain A, which exhibits a liver threefold larger than other strains. HMG-CoA reductase activity, the rate-limiting enzyme in cholesterol synthesis, is not diminished when Strain A is switched from a low- to high-fat and cholesterol diet, suggesting that cholesterol synthesis is not down-regulated (Ref. 20; and Paigin, B., Chen, H., and Billheimer, H., unpublished observations). The combination of exogenous and endogenous cholesterol coupled with the lower phospholipid content of the liver may lead to the extreme accumulation of liver cholesterol in Strain A.

Genetic variants in plasma lipids, basal levels and response to high-fat diet. Many aspects of plasma lipids are similar between humans and mice, such as apolipoprotein and lipid composition of the lipoprotein particles and the responses observed with dietary manipulation. Therefore, the mouse model, with its unique advantages for dissecting genetic determinants, may be useful in identifying heritable factors that control lipid metabolism. Differences between humans and mice do occur, such as in the relative proportions of atherogenic lipoprotein particles. In humans, LDL predominates and in mice VLDL predominates (21), presumably because mice lack cholesterol ester transfer activity (22).

The three major observations of this study, which may warrant further investigation, were made when animals were challenged with the high-fat and high-cholesterol diet. The first observation is the lack of change in plasma triglyceride levels in some animals when fed the high-fat diet. A decrease in fasting plasma triglyceride levels would be expected as a result of the dietary fat because long-chain fatty acids inhibit *de novo* lipogenesis (23). This drop occurred in two strains, SM and DBA/2. Srivastava *et al.* (24) also reported similar results; a decrease in plasma triglyceride levels in DBA/2 animals and no change from basal levels in strains C57BL/6, C57/L, SWR and C3H/He. They did, however, see a significant decrease in plasma triglyceride levels in AKR mice when switched from a low fat diet to a semi-synthetic diet containing 21% fat and 2% cholesterol with no cholic acid. The difference in

response among mouse strains to dietary fat and cholesterol intake on plasma triglyceride levels needs to be studied further.

The second interesting difference among the strains was the hyper- or hyporesponsiveness observed in plasma cholesterol levels. This difference in response to a high-fat/high-cholesterol diet has been observed in other species such as primates, rabbits and rats (25) as well as in mice (24,26). As in other species, the changes in plasma cholesterol are due to an increase in cholesterol contained in the VLDL and LDL fraction. In our studies, strains C57L and SWR were hyper-responders and AKR and SJL were hyporesponders. These strains are progenitors of recombinant inbred sets, C57L × AKR and SWR × SJL. The responsiveness of combined VLDL and LDL to dietary lipid in these recombinant inbred sets could be used to identify the genetic determinants involved.

The third observation of interest was the failure of the HDL-C levels to decrease in SM mice fed the semi-synthetic atherogenic diet. A hallmark of susceptibility for aortic lesion development in mice studied thus far is a marked reduction in plasma HDL-C levels (17,18). SM mice, which were susceptible to aortic lesions, had basal HDL-C levels of 42 ± 5 mg/dL and of 59 ± 5 mg/dL after four weeks of consuming the atherogenic diet. Normally, the lack of change or slight increase in HDL-C levels upon high-fat feeding is associated with atherosclerosis-resistant strains of mice such as C3H or A.

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Adipose Glycerolipid Formation: Effect of Nutritional and Hormonal States

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The potential of glycerolipid formation from *sn*-glycerol-3-phosphate (GP) and 2-monoacylglycerol (MG) was studied in adipose microsomal fractions under various nutritional and hormonal states. Glycerolipid formation from GP was followed in the presence of [^{14}C]glycerol-3-phosphate and palmitoyl-CoA and was assayed by measuring the formation of butanol-soluble product, consisting mainly of [^{14}C]phosphatidate. Glycerolipid formation from MG was determined in the presence of 2-monooleyl glycerol and [^{14}C]palmitoyl-CoA, and was estimated by the formation of both [^{14}C]di- and triacylglycerol. Glycerolipid formation from GP was decreased significantly during food deprivation, in experimental diabetes, in the presence of lipolytic hormone, and during aging. Glycerolipid formation from MG did not change under these conditions and continued at the same rate as observed in control animals. The rate of glycerolipid formation from GP was 7–20 times greater than from MG in the various fat depots. Measurement of the adipose monoacylglycerol concentration did not show any correlation with the glycerolipid formation from MG. The studies suggest that glycerolipid formation from MG is active in various fat depots, and is substantial when glycerolipid formation *via* GP is impaired.
Lipids 28, 607-612 (1993).

It is now well known that fatty acid esterification in adipose tissue is mediated by the stepwise acylation of either *sn*-glycerol-3-phosphate (1,2), dihydroxyacetone phosphate (3) or 2-monoacylglycerol (4). Comparisons of the relative rates of esterification from various precursors indicate that the *sn*-glycerol-3-phosphate pathway is the major route of glycerolipid synthesis in adipose tissue (5).

In several studies, the changes in glycerolipid synthesis from *sn*-glycerol-3-phosphate were shown to be positively correlated with the rate of triacylglycerol accumulation in adipocytes (6–8). However, such a relationship was not observed in animals over 60 d of age. Adipose glycerolipid synthesis from *sn*-glycerol-3-phosphate in these animals showed a more than 100% decrease compared to young animals, in spite of the increase in triacylglycerol accumulation (8). This suggested that alternative metabolic routes other than the *sn*-glycerol-3-phosphate pathway may be operative in the deposition of triacylglycerol in older adipocytes. Another observation that suggested such a possibility was the stimulation of fatty acid esterification in intact adipocytes by catecholamines (9), when inactivation of several enzymes of the *sn*-glycerol-3-phosphate pathway is known to occur (10,11). Previous studies from our laboratory have shown that glycerolipid synthesis by the monoacylglycerol (MG) pathway is active in aged rats, in spite of the significant decrease in glycerolipid formation by the *sn*-glycerol-3-phosphate pathway (12).

The presence of the MG pathway in adipose tissue has been known for 20 years (4). Comparisons of glycerolipid formation *via* glycerophosphate and monoacylglycerol have indicated that, in hamster adipose tissue, the MG pathway is quite active, *i.e.,* shows about 40% of the activity of the *sn*-glycerol-3-phosphate pathway. Moreover, 2-monooleylglycerol ether, a 2-monoolein analogue which was used to measure the contribution of the MG pathway, inhibited glycerolipid formation *via* the *sn*-glycerol-3-phosphate pathway in both intact adipocytes and in a cell-free system (4,13,14). These observations led Johnston and coworkers (13,14) to propose that the MG pathway may play a significant role in adipose glycerolipid synthesis. In contrast, Dodds *et al.* (5,15) and Christie and Hunter (16) showed an insignificant contribution (<10% of the glycerophosphate pathway) of the MG pathway to glycerolipid synthesis in rat adipose tissue. Thus, the significance of the MG pathway in adipose glycerolipid formation is still not quite clear, although this pathway is considered the major route of glycerolipid synthesis in intestine (17) and in neonatal rat liver (18).

The present study was undertaken to determine the contribution of the MG pathway to glycerolipid formation in adipose tissue under various experimental conditions which are known to impair glycerolipid synthesis from *sn*-glycerol-3-phosphate. This approach was thought to provide information on the physiological importance of the MG pathway in adipose tissue.

MATERIALS AND METHODS

Materials. [U-^{14}C]*sn*-glycerol-3-phosphate (spec. radioactivity, 161 mCi/mmol) and [1-^{14}C]palmitoyl-CoA (spec. radioactivity, 54 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 2-Monoolein and 2-monooleyl glycerol ether were purchased from Serdary Research Laboratories (Port Huron, MI). Other monoacylglycerols were obtained from Nu-Chek-Prep (Elysian, MN). Palmitoyl-CoA, ATP, coenzyme A, dithiothreitol, BSTFA (*bis*[trimethylsilyl]trifluoroacetamide), pyruvate kinase, glycerol kinase, lactate dehydrogenase (LDH), phosphoenolpyruvate, nicotinamide adenine dinucleotide reduced form (NADH), triethanolamine, hexokinase (Type VII from baker's yeast), glucose-6-phosphate dehydrogenase (Type VII from baker's yeast) and streptozotocin were purchased from Sigma Chemical Co. (St. Louis, MO).

Male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were placed in an air-conditioned animal facility maintained on a 12-h dark (6 a.m.–6 p.m.)/12-h light (6 p.m.–6 a.m.) schedule. Animals were fed laboratory chow (Ralston Purina Laboratory, St. Louis, MO) *ad libitum.* After sedation (by

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Abbreviations: BSA, bovine serum albumin; BSTFA, *bis*(trimethylsilyl)trifluoroacetamide; CoA, coenzyme A; LDH, lactate dehydrogenase; MG, monoacylglycerol; MGAT, monoacylglycerol acyltransferase; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide reduced form; Tris, tris(Hydroxyethyl)-aminomethane.

exposing to CO_2), rats were sacrificed between 8 and 10 a.m. by decapitation.

Preparation of subcellular fractions. The adipose tissue microsomal fractions were prepared from epididymal fat pads from a single animal or pooled from two animals, as described previously (19). In some experiments, in addition to epididymal fat pads, the subcutaneous and perirenal fat pads were used. Adipose tissues from different sources were homogenized with a Teckmar Tissuemizer (Teckmar Company, Cincinnati, OH) with 3 vol of cold Medium A (0.25 M sucrose/1 mM tris(hydroxymethyl)-aminomethane (Tris) (pH 7.5)/1 mM ethylenediaminetetraacetic acid/1 mM dithiothreitol). The homogenate was further processed to isolate microsomal fractions as described previously (19).

Metabolic studies. To investigate the acute effects of hormones on glycerolipid formation from *sn*-glycerol-3-phosphate and 2-monoacylglycerol, epididymal adipose fragments (30–40 mg tissue) were incubated in the presence of either insulin (1,2 milliunit/mL) or 6 μM norepinephrine for 1 h at 37°C in 8 mL Krebs-Ringer-bicarbonate containing 4% bovine serum albumin (BSA), 10 mM glucose and half the recommended amount of Ca^{2+}. All incubations were carried out in a shaking water bath, under an atmosphere of O_2/CO_2 (19:1), in polyethylene vials. Following this incubation, the fragments were washed thoroughly in warm Medium A to remove adherent albumin and hormones. The washed fragments were then homogenized with cold Medium A to isolate microsomal fractions (19), which were used to measure glycerolipid formation from both *sn*-glycerol-3-phosphate and 2-monoacylglycerol. The incubation mixture from metabolic studies was stored frozen at −80°C and used at a later date for the determination of glycerol, which was taken as a measure of lipolytic activity of adipose tissue in the presence of different hormones.

Effect of fasting, refeeding and streptozotocin-induced diabetes on glycerolipid formation. Since the effect of fasting, refeeding and diabetes on glycerolipid formation from 2-monoacylglycerol was not known, we investigated the effect of these experimental conditions on glycerolipid formation from both *sn*-glycerol-3-phosphate and 2-monoacylglycerol. In these studies, rats were deprived of food for 48 h and refed for 48 h following a 48-h fast (48-h fast and 48-h refed group, see Tables 1 and 3). Diabetes was induced in animals by injecting 60 mg/kg of streptozotocin intramuscularly. Control rats received saline. After 72 h, the severity of diabetes in the experimental animals was monitored by measuring plasma glucose concentrations.

Enzyme assays and identification of the reaction products. In the present studies, glycerolipid formation from *sn*-glycerol-3-phosphate and 2-monooleylglycerol was measured in the presence of palmitoyl-CoA, which served as acyl donor for both enzymatic reactions. Initial studies were conducted to determine the optimal concentrations of substrates and cofactors for these reactions. Under the assay conditions, glycerolipid formation from both *sn*-glycerol-3-phosphate and 2-monooleylglycerol was linear with time and the amount of microsomal protein added. Under all our experimental conditions, initial enzyme reaction rates were measured and the appropriate substrates and enzyme blanks were included to estimate the true enzyme activity.

Glycerolipid formation from 2-monooleylglycerol was measured as described previously (20). In a final volume of 1 mL, the reaction mixture contained 24 mM Tris/HCl buffer, pH 7.5, 50 mM KCl, 8.0 mM $MgCl_2$, 0.25 mM monooleylglycerol, 0.75 mM dithiothreitol, 25μ M [^{14}C]-palmitoyl-CoA (0.024 μCi), 20 μL of phospholipid mixture containing 15 μg of phosphatidylcholine and 15 μg of phosphatidylserine, and 1.25 mg of BSA. The reaction was started by the addition of the adipose microsomal protein (80–140 μg). The incubation was carried out at 37°C and was linear with time for 15 min. The formation of both [^{14}C]di- and triacylglycerol was taken as a measure of enzyme activity. In the absence of monooleyl glycerol, very little (less than 5% of total) incorporation of [^{14}C]-palmitoyl-CoA into diacylglycerol and triacylglycerol occurred. The enzyme reaction stopped at zero time was subtracted as blank. The specific activity of the MG pathway was calculated on the basis of radioactivity present in the diacylglycerol fraction plus 1/2 of the radioactivity in the triacylglycerol fraction, as two moles of palmitoyl-CoA were required to form triacylglycerol from 2-monoacylglycerol.

The acylation of *sn*-glycerol-3-phosphate in the presence of palmitoyl-CoA was studied as described previously by Rider and Saggerson (21), with slight modifications. In a final volume of 0.70 mL, the reaction mixture contained 24 mM Tris/HCl buffer, pH 7.5, 50 mM KCl, 0.84 mM *sn*-glycerol-3-phosphate containing [^{14}C]glycerol-3-phosphate (0.1 μCi), 0.8 mM dithiothreitol, 65 μM palmitoyl-CoA and 1.75 mg of BSA. The reaction was started with 15–20 μg microsomal protein and was linear with time for 4 min. Incubation was under air at 30°C in a shaking water bath. Incorporation of [^{14}C]glycerol-3-phosphate into butanol-soluble products, mainly radioactive phosphatidate, was taken as a measure of enzyme activity.

The radioactive lipids formed from 2-monoacylglycerol and [^{14}C]palmitoyl-CoA were extracted as described by van den Bosch and Vagelos (22), and the procedure of Daae and Bremer (23) was followed to isolate the butanol-soluble products formed from [^{14}C]glycerol-3-phosphate and palmitoyl-CoA. The lipids were dried under N_2 and dissolved in 0.5 mL of chloroform/methanol (2:1, vol/vol). Samples were applied in a volume of 0.1 mL and separated on thin-layer plates coated with Silica G (E. Merck, Darmstadt, Germany). The lipid classes were identified by running appropriate standards in an adjacent lane.

Phosphatidate was separated using the solvent system chloroform/methanol/3M-NH_3 (65:38:8, by vol) and chloroform/methanol/acetic acid/water (50:25:8:4, by vol) (24,25). Neutral lipids were separated with hexane/diethyl ether/acetic acid (73:25:2, by vol) (26). The lipids were localized by exposure of the plates to iodine vapors. After sublimation of I_2 at room temperature, appropriate areas from the plates were scraped directly into scintillation vials containing 10 mL of CytoScient (ICN Biochemicals, Irvine, CA). The radioactivity was measured in a Packard (Packard Instrument Company, Meriden, CT) Tricarb 480 scintillation counter.

Analysis of total lipids, monoacylglycerols, glycerol and glucose. Total lipid and monoacylglycerol concentrations were determined in epididymal tissue during fasting and refeeding, in diabetic animals and from adipose fragments incubated in the presence of various hormones. Tissue lipids were extracted by the Folch procedure (27), and the

total lipid concentration was measured spectrophotometrically according to Barnes and Blackstack (28). The monoacylglycerol concentration was measured according to Arner and Ostman (29). For this purpose, monoacylglycerols were separated from other lipids by thin-layer chromatography on silica gel 60 with hexane/diethyl ether/acetic acid (73:25:2, by vol) as solvent system. The silica gel scrapings containing monoacylglycerols were extracted twice with 5 mL of chloroform/methanol (4:1, vol/vol) (29). The combined chloroform/methanol extracts were dried under N_2 and the monoacylglycerols were hydrolyzed at 70°C for 30 min in the presence of ethanolic KOH (29). Following addition of 1.5 mL of 0.05M $MgSO_4$, an aliquot of the supernatant was taken for the enzymatic determination of glycerol (30). In some experiments, we also determined the adipose monoacylglycerol composition. In these experiments, adipose monoacylglycerols, purified as described above, were derivatized with 100 μL BSTFA at 75°C for 45 min in the presence of pyridine (31). The contents were dried under N_2, and the derivatized monoacylglycerols were dissolved in 50 μL of methylene chloride. Two-microliter aliquots (in duplicate) were injected into the gas chromatograph (Hewlett-Packard, Kennet Square, PA; Model 5890, Series II, equipped with a 3396A integrator) and separated on a capillary column (DB1, 30 m × 0.53 mm i.d. 5 μm thickness, J&W PN 1235-1035, obtained from J&W Scientific, Folsom, CA) and analyzed using a flame-ionization detector. Gas chromatographic conditions were as follows. Oven temperature, 280°C, held for 3 min, then increased to 290°C at a rate of 1°C/min. The injector and detector temperatures were maintained at 280 and 320°C, respectively. The carrier gas (helium) flow was at 22 mL/min. The amounts of monoacylglycerols present in samples were determined relative to peak heights of monoacylglycerol standards analyzed at the same time under the same conditions.

Glycerol concentrations were determined spectrophotometrically by using the assay procedure of Thorner and Paulus (30). The reaction velocity was measured in a coupled system through glycerol kinase, pyruvate kinase and LDH. Glycerol was estimated on the basis of the conversion of NADH into nicotinamide adenine dinucleotide $(NAD)^+$ as monitored at 340 nm. Plasma glucose concentrations from diabetic and control animals were measured by the "IL Test"™ (Instrumentation Laboratory Microcentrifugal Analyzer) (32). Glucose concentrations were measured in a coupled system through hexokinase and glucose 6-phosphate dehydrogenase, and quantified based on the conversion of NAD^+ to NADH as monitored at 340 nm.

RESULTS AND DISCUSSION

In adipose tissue, monoacylglycerols are formed during hydrolysis of triacylglycerols by both hormone-sensitive lipase (33) and lipoprotein lipase (34). In spite of the presence of monoglyceride lipase (33), the monoacylglycerols, which can be cytotoxic (35), accumulate in adipose tissue (36). Recycling of monoacylglycerols to diacylglycerols is mediated by the monoacylglycerol acyltransferase (MGAT), an initial reaction of the MG pathway.

Besides being important in glycerolipid formation, the MG pathway may also participate in other aspects of adipose lipid metabolism, including the formation of the second messenger diacylglycerol (37), the proper functioning of lipoprotein lipase by interfering with the product inhibition of this enzyme by monoacylglycerols (34), as well as the protection of adipose tissue against the cytotoxic effects of monoacylglycerols (35). In the present investigation, we have directed our attention mainly on the role of the MG pathway in adipose glycerolipid synthesis and compared its activity with that of the *sn*-glycerol-3-phosphate pathway (Table 1). The rates of glycerolipid formation from *sn*-glycerol-3-phosphate were several times greater than those from 2-monoacylglycerol. This was evident under all the experimental conditions investigated

TABLE 1

Effect of Nutritional and Hormonal States on Adipose Glycerolipid Formation[a]

Nutrition or hormonal state	Body weight (g)	Adipose weight (g)	Glycerolipid formation[b] from		Glycerol[c] μmoles/g	Plasma glucose mg%
			sn-glycerol-3-phosphate	2-monoacylglycerol		
Expt. 1, nutritional						
Control (8)	216 ± 10.17	1.49 ± 0.22	68.96 ± 16.26	4.30 ± 0.23	nd[e]	nd
48-h fast (8)	175 ± 8.60	0.75 ± 0.20	39.81 ± 13.84[d]	5.15 ± 1.30	nd	nd
48-h fast and 48-h refeed (8)	184 ± 9.88	0.99 ± 0.20	59.33 ± 8.52	5.40 ± 1.11	nd	nd
Expt. 2, hormonal						
Control (8)	249 ± 8.5	1.22 ± 0.39	63.83 ± 12.29	5.53 ± 0.76	2.47 ± 0.92	nd
Insulin (9)	248 ± 16.5	1.15 ± 0.31	59.98 ± 9.22	4.92 ± 0.44	2.14 ± 0.85	nd
Norepinephrine (9)	247 ± 8.9	1.19 ± 0.42	42.33 ± 6.27[d]	5.82 ± 0.54	9.48 ± 0.46[f]	nd
Expt. 3, hormonal						
Control (9)	259 ± 8.0	1.34 ± 0.24	63.99 ± 7.52	5.06 ± 0.91	nd	142 ± 20
Diabetes (10)	244 ± 16	0.93 ± 0.22	43.32 ± 8.3[d]	5.16 ± 0.48	nd	382 ± 51

[a]Epididymal adipose microsomal fractions were used. Expt., experiment.
[b]Glycerolipid formation from *sn*-glycerol-3-phosphate and 2-monoacylglycerol was measured as described in Materials and Methods, and the rates of glycerolipid formation are expressed as nmoles of lipid formed per min per mg microsomal protein. Each value is the mean ± SD from the number of animals referred to in parentheses.
[c]Glycerol concentration in the incubation medium is expressed as μmoles of glycerol released per g of adipose tissue.
[d]Significantly different from control and refed animals, $P < 0.05$.
[e]nd, Not determined.
[f]Significantly different from control and insulin group, $P < 0.001$.

TABLE 2

Adipose Glycerolipid Formation in Various Fat Depots from Young and Old Animals[a]

Age (wk)	Body weight (g)	Source of adipose tissue	Glycerolipid formation[b] from *sn*-glycerol-3-phosphate (A)	Glycerolipid formation[b] from 2-monoacylglycerol (B)	A/B
6-7	223 ± 6.7	Epididymal	76.34 ± 9.96	5.27 ± 0.91	14.48
		Perirenal	70.74 ± 13.31	3.08 ± 0.40	22.96
		Subcutaneous	25.14 ± 4.34[c]	3.17 ± 0.14	7.93
13-14	356 ± 6.83	Epididymal	47.68	4.48 ± 0.14	10.64
		Perirenal	41.90 ± 14.73[d]	3.67 ± 0.14	11.41
		Subcutaneous	14.81 ± 1.01[d]	2.12 ± 1.48	6.98

[a]Adipose microsomal preparations from epididymal, perirenal and subcutaneous fat pads were used.
[b]The rates of glycerolipid formation from *sn*-glycerol-3-phosphate and 2-monoacylglycerol are expressed as nmoles of products formed per min per mg protein. Each value is the mean ± SD from 4-6 experiments, using 6 old and 19 young animals, except for epididymal fat tissue from old animals, where the data from two experiments were pooled.
[c]Significantly different from other fat depots in the same group of animals, $P < 0.001$.
[d]Significantly different from young animals, $P < 0.05$.

so far. The fasting of animals for 48 h did cause a significant reduction in the formation of glycerolipids from *sn*-glycerol-3-phosphate, and refeeding of fasted animals for 48 h partially retrieved this activity (Experiment 1), consistent with our earlier observations (38). However, glycerolipid formation from monoacylglycerol continued at the same rate as control animals during a fasting and refeeding cycle.

Microsomal fractions prepared from adipose tissue fragments pretreated with norepinephrine also showed a significant reduction in the formation of glycerolipids from *sn*-glycerol-3-phosphate (Experiment 2). Such an effect of this lipolytic hormone was also noted by Saggerson and co-workers (10,11) and was attributed to the inactivation of *sn*-glycerol-3-phosphate acyltransferase and Mg^{2+}-dependent phosphatidate phosphohydrolase. The glycerolipid formation from 2-monoacylglycerol continued at a normal rate in the presence of lipolytic hormone, in spite of the significant decrease in the synthesis of glycerolipids from *sn*-glycerol-3-phosphate.

The tissue fragments incubated with insulin did not show any effect on the glycerolipid formation from both 2-monoacylglycerol and *sn*-glycerol-3-phosphate (Experiment 2). However, the microsomal fractions obtained from diabetic animals showed a significant reduction in the rates of glycerolipid formation from *sn*-glycerol-3-phos-

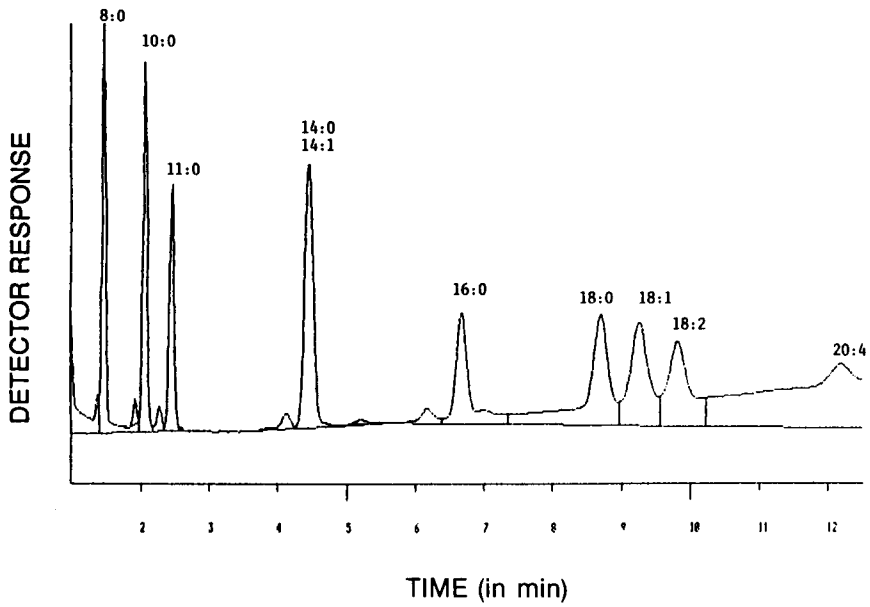

FIG. 1. Gas chromatographic (GC) separation of adipose monoacylglycerols. Adipose tissue monoacylglycerols were isolated and derivatized as described in Materials and Methods. A 2-μL aliquot (containing 250 ng of each monoacylglycerol species) was injected into the gas chromatograph and analyzed by flame-ionization detection. GC conditions: capillary column, 30 m × 0.53 mm i.d., 5 μm thickness, phase DB-1, and temperature program as described in Materials and Methods.

phate as noted previously (39), suggesting that insulin has a role in maintaining the optimal activity of glycerolipid formation from *sn*-glycerol-3-phosphate. Again, glycerolipid formation from 2-monoacylglycerol in diabetic animals continued at the same rate as in control animals. Thus, glycerolipid formation from 2-monoacylglycerol appears more resistant to changes in the hormonal and nutritional status of the animals, whereas glycerolipid formation *via sn*-glycerol-3-phosphate seems more responsive to changes in environmental conditions.

Besides in epididymal tissue, the MG pathway was also found operative in perirenal and subcutaneous adipose tissue of young and old animals (Table 2). However, the contribution of this pathway to glycerolipid formation differed from one adipose depot to the other. Glycerolipid formation from *sn*-glycerol-3-phosphate decreased in all the fat depots as the animals grew older, as noted previously (8), without any significant changes occurring in the rates of glycerolipid formation of 2-monoacylglycerol.

Monoacylglycerols serve as substrates for various enzymatic reactions, including MGAT, monoacylglycerol lipase and monoacylglycerol kinase. Therefore, in some experiments we measured the adipose monoacylglycerol concentration in rats under various hormonal and nutritional states (Table 3). As noted earlier (36), the accumulation of monoacylglycerols in adipose tissue was apparent and was increased further when adipocyte lipolysis was stimulated, *i.e.*, during fasting, refeeding and in diabetic animals, and was decreased when the lipolytic process was inhibited in the presence of insulin (40). Although changes in the monoacylglycerol content did not directly affect MGAT activity measured *in vitro*, the observed increases in the MG content during lipolysis could increase the rate of flux of these lipids *via* MGAT and enhance the recycling of endogenous monoacylglycerols by the MG pathway.

Our data show (Table 3 and Fig. 1) that rat adipose tissue contains both short- and long-chain monoacylglycerols, with some minor changes occurring under different hormonal and nutritional conditions. The most notable change in the composition of monoacylglycerol was apparent in the adipose tissue fragments which were incubated at 37°C for 1 h. The incubated adipose tissue contained very high amounts of 20:4 monoacylglycerols (as high as 80% of total) compared to the adipose tissue fragments, which were subjected to lipid extraction immediately following the excision of adipose tissue (in fed, fasted, refed and diabetic animals). The reason for the increase in 20:4 monoacylglycerol content, during incubation of adipose tissue at 37°C, is presently not clear. However, this increase does not seem to be hormonally related, since it occurred in all samples regardless of the presence of hormones. In addition to 10 monoacylglycerol species identified, the rat adipose tissue also contained three minor peaks of unidentified monoacylglycerols, which were eluted between 8:0 and 14:0 monoacylglycerols (data not reported).

In conclusion, a comparison of adipose glycerolipid formation from *sn*-glycerol-3-phosphate and from 2-monoacylglycerol provided evidence that the *sn*-glycerol-3-phosphate pathway is more active and responds more readily to changes in the hormonal and nutritional status of the animal. The maintenance of the optimal rates of glycerolipid formation from 2-monoacylglycerol, during impairment of glycerolipid formation from

TABLE 3

Effect of Nutritional and Hormonal States on Total Lipid and Monoacylglycerol Concentrations[a]

Nutritional and hormonal state	Total lipid μmoles/g	Monoacylglycerols nmoles/g	% Distribution of Monoacylglycerols of different chain lengths[b]							
			8:0	10:0	11:0	14:0, 14:1	16:0	18:1	18:0	18:2	20:4
Control (6)	442 ± 103	493 ± 118[c,d]	9.6 ± 5.0	8.4 ± 3.5	9.8 ± 4.6	4.2 ± 1.9	7.4 ± 4.2	5.2 ± 2.6	23.9 ± 14.2	15.2 ± 6.6	18.2 ± 15.0
48-h fast (4)	416 ± 10	868 ± 25[c,d]	10.2 ± 2.9	9.0 ± 2.5	11.2 ± 3.6	4.5 ± 1.8	12.7 ± 4.4	8.9 ± 6.6	28.6 ± 11.2	6.2 ± 5.7	8.4 ± 3.5
48-h fast + 48-h refeed (4)	466 ± 42	1607 ± 622[d,e]	2.6 ± 4.4	2.8 ± 3.3	4.3 ± 3.6	2.6 ± 2.2	11.57 ± 5.4	3.8 ± 4.9	27.7 ± 11.7	30.5 ± 4.4	14.2 ± 3.9
Diabetes (4)	369 ± 38	611 ± 115[d]	5.5 ± 5.7	1.6 ± 2.1	9.4 ± 4.3	3.1 ± 1.5	7.2 ± 3.1	1.9 ± 1.9	30.4 ± 11.6	25.9 ± 16.8	14.9 ± 8.0
Control (4)	378 ± 32	676 ± 112[f]	0.4 ± 0.6	0.4 ± 0.5	0.5 ± 0.6	5.3 ± 4.0	4.0 ± 4.0	2.0 ± 2.0	2.1 ± 1.5	4.8 ± 1.9	80 ± 4.0
Norepinephrine (4)	388 ± 46	826 ± 157[e,f]	0.1 ± 0.1	1.9 ± 1.5	3.1 ± 1.7	1.6 ± 1.4	2.6 ± 1.8	2.3 ± 0.6	2.6 ± 1.8	2.7 ± 1.8	82.6 ± 3.6
Insulin (4)	397 ± 12	104 ± 30[f,g]	2.7 ± 2.2	3.2 ± 1.7	5.4 ± 2.7	2.8 ± 1.9	3.9 ± 1.9	2.8 ± 2.3	3.4 ± 3.6	9.5 ± 2.3	68.2 ± 5.6

[a]Epididymal adipose tissue fragments were used to measure total lipid and monoacylglycerol concentrations and composition as described in Materials and Methods.
[b]Composition of various monoacylglycerols was determined by capillary gas chromatography as described in Materials and Methods.
[c]Each value is the mean ± SD from the number of experiments indicated in parentheses. For each experiment, adipose fragments from two rats (details are indicated in Table 1) were used and analyzed in duplicate.
[d]After sacrificing animals, epididymal adipose tissue fragments were immediately placed in 10 mL chloroform/methanol (2:1, vol/vol) for lipid extraction and analysis of total lipid and monoacylglycerols.
[e]Significantly different from control animals, $P < 0.05$.
[f]Epididymal adipose fragments were incubated for 1 h at 37°C in the presence of insulin or norepinephrine before lipid extraction and analyses of total lipids and monoacylglycerols.
[g]Significantly different from control animals, $P < 0.01$.

sn-glycerol-3-phosphate, suggests that the increased rates of fatty acid esterification (9) and triacylglycerol accumulation (8) that have been noted previously, may be partly mediated through the MG pathway.

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Multiple Inhibitory Effects of Garlic Extracts on Cholesterol Biosynthesis in Hepatocytes

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Exposure of primary rat hepatocytes and human HepG2 cells to water-soluble garlic extracts resulted in the concentration-dependent inhibition of cholesterol biosynthesis at several different enzymatic steps. At low concentrations, sterol biosynthesis from [^{14}C]acetate was decreased in rat hepatocytes by 23% with an IC_{50} (half-maximal inhibition) value of 90 μg/mL and in HepG2 cells by 28% with an IC_{50} value of 35 μg/mL. This inhibition was exerted at the level of hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) as indicated by direct enzymatic measurements and the absence of inhibition if [^{14}C]mevalonate was used as a precursor. At high concentrations (above 0.5 mg/mL), inhibition of cholesterol biosynthesis was not only seen at an early step where it increased considerably with dose, but also at later steps resulting in the accumulation of the precursors lanosterol and 7-dehydrocholesterol. No desmosterol was formed which, however, was a major precursor accumulating in the presence of triparanol. Thus, the accumulation of sterol precursors seems to be of less therapeutic significance during consumption of garlic, because it requires concentrations one or two orders of magnitude above those affecting HMG-CoA reductase. Alliin, the main sulfur-containing compound of garlic, was without effect itself. If converted to allicin, it resulted in similar changes of the sterol pattern. This suggested that the latter compound might contribute to the inhibition at the late steps. In contrast, nicotinic acid and particularly adenosine caused moderate inhibition of HMG-CoA reductase activity and of cholesterol biosynthesis suggesting that these compounds participate, at least in part, in the early inhibition of sterol synthesis by garlic extracts.
***Lipids 28,* 613–619 (1993).**

Long-term exposure of rats to water-soluble garlic extracts has been reported to result in decreased serum cholesterol and triglyceride levels (1). Similar observations were reported for rabbits with and without feeding cholesterol (2,3) as well as for other species (see Ref. 4). Recently, significant reduction in serum cholesterol after long-term intake of garlic powder tablets has been found for humans in double-blind crossover studies (5,6). Little is known as yet about the mechanism(s) and the active principle(s) responsible for these effects.

Primary cultures of rat hepatocytes provide a suitable model system for studies on the physiological regulation and pharmacological modulation of cholesterol biosynthesis and metabolism (7–13). Likewise, HepG2 cells have been used for characterizing hepatic cholesterol metabolism and its inhibition (14–16). Using rat hepatocyte cultures we have recently described inhibition of cholesterol biosynthesis by water-soluble garlic extracts (17). In the present study, we have carefully analyzed the concentration dependence of these effects and report on the interaction at different points during the biosynthetic pathway of cholesterol depending upon the concentration of the garlic extracts. In addition, the effect of garlic extracts and of some constituents on cholesterol biosynthesis in rat hepatocytes was compared with that in HepG2 cells.

MATERIALS AND METHODS

Materials. The garlic powder used (corresponding to Kwai®/Sapec®, Lichtwer Pharma, Berlin, Germany) is prepared immediately after harvesting of garlic cloves from central China. The garlic of this region is characterized by a very high content of sulfur-containing components. The water is removed from the fresh garlic cloves by careful air drying. The other components (such as alliin and the enzyme alliinase) are maintained in concentrated form in the garlic powder. The powder is standardized to an alliin content of 1.3% (18,19) and a capacity for liberation of allicin of 0.6%. The garlic powder, as well as pure, synthetic alliin (18) were provided by Lichtwer Pharma GmbH. These substances were kept dry at 4°C. Mevastatin, triparanol and ketoconazole were kind gifts from Dr. H.J. Kempen (TNO, Leiden, Netherlands).

[U-^{14}C]Acetate sodium-salt (1.9 GBq/mmol, 51.3 mCi/mmol) and RS-[2-^{14}C]mevalonic acid dibenzylethylenediamine salt (2.1 GBq/mmol, 56.7 mCi/mmol) for cholesterol biosynthesis, as well as [1-^{14}C]oleic acid (1.95 GBq/mmol, 52.6 mCi/mmol) and [4-^{14}C]cholesterol (2.2 GBq/mmol, 59.4 mCi/mmol) for the determination of enzyme activities were obtained from Amersham/Buchler (Braunschweig, Germany).

Collagenase (0.225 U/mg) was from Boehringer (Mannheim, Germany) and was used only from batches which proved suitable for the isolation of hepatocytes in our laboratory. Newborn calf serum was obtained from Sebio (Walchsing, Germany) or Serva (Heidelberg, Germany); Williams Medium E was from Flow Laboratories (Meckenheim, Germany). The Extrelut® 20-columns were from Merck (Darmstadt, Germany). All other chemicals were from Boehringer, Merck or Sigma (Munich, Germany).

Animals. Male Sprague-Dawley rats (220–270 g) were used as hepatocyte donors. They were kept in a controlled 12-h light/dark cycle on a standardized diet of Alma® H 1003 (Botzenhardt, Kempten, Germany) and tap water *ad libitum.*

Isolation and cultivation of rat hepatocytes. Rat hepatocytes were isolated according to the two-step collagenase perfusion technique previously described (20). Viability of the cells averaging to 93.2 ± 3.1% was routinely checked by staining with trypan blue.

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Abbreviations: HMG-CoA reductase, hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34); IC_{50}, the concentration for half-maximal inhibition; LDH, lactate dehydrogenase (EC 1.1.1.27); SI-TLC, silver-ion thin-layer chromatography.

Dedication: This article is dedicated to Prof. Dr. D. Mecke on the occasion of his 60th birthday.

The isolated hepatocytes were suspended in Williams Medium E containing 10% newborn calf serum, 2 mM glutamine, penicillin (50 units/mL), streptomycin (50 μg/mL) and 10^{-7} M dexamethasone (17). They were seeded in 2 mL of culture medium at a density of 1.25×10^5 cells/cm^2 into Petri dishes (ϕ 60 mm) precoated with a thin film of collagen (21) and were incubated at 37°C, 90% humidity and 7% CO_2. From 2 h on, serum-free medium was used.

Cultivation of HepG2 cells. The human hepatoma cell line HepG2 was maintained as monolayers as described (22). Confluent cultures were detached with 0.2% trypsin, suspended in medium containing 0.2% trypsin inhibitor and split 1:3 once a week. The cells were used between passages 40–70 (our laboratory). Incubations with garlic extracts were performed in the same serum-free medium used for hepatocytes.

Extraction of garlic powder. Garlic powder stored under dry conditions was extracted with doubly distilled water as described (17), carefully (<30°C) evaporated to dryness and redissolved in Williams Medium E (5 mL/g powder; initial weight). This stock solution was further diluted with Williams Medium E. All concentration data relate to the initial amount of garlic powder per extraction volume, since the amount of the dissolved material (approximately 80%) was not determined. Control media were made using doubly distilled water instead of the garlic extract.

Pure alliin was dissolved directly in Williams Medium E (1 mg/mL) and sterilized by filtration. Dilutions were always made with Williams Medium E. For preincubation with alliinase, alliin (1.66 mg/mL) was directly dissolved in a garlic extract of 5 mg/mL) and was incubated at room temperature for 20 min. These conditions should lead to the complete conversion of all added alliin to allicin (18). The mixture was then diluted 1:100 with Williams Medium E.

Incubation of the primary cultures with the extracts. Eighteen hours after inoculation of the hepatocyte cultures the medium was removed and fresh medium containing [^{14}C]acetate (18.5 KBq/mL; 0.5 μCi/mL), together with the appropriate dilutions of the garlic extracts or compounds to be tested, was added. After incubation at 37°C for 2 h, the medium was removed and the cell layer was washed twice with saline and scraped into 2 mL distilled H_2O. The cells were homogenized by sonication (20 s, grade 3, Ref. 20). In several experiments [^{14}C]-acetate was replaced by [^{14}C]mevalonate (9 KBq/mL, 0.24 μCi/mL).

Determination of acetate incorporation into and separation of nonsaponifiable neutral lipids. The incorporation of [^{14}C]acetate into nonsaponifiable neutral lipids was determined as described using Extrelut®-columns (large-pore kieselgur) for efficient separation (17). The neutral lipophilic nonsaponifiable substances were eluted with *n*-heptane (17). The precursors such as [^{14}C]acetate or [^{14}C]-mevalonate are retained on the column to more than 99%. For measurements of incorporation, the eluate was collected directly in scintillation vials and measured in the scintillation counter after addition of 10 mL of Ultima Gold® (Packard, Merident, CT). The yield of the elution step was 92% (86–95%). Recovery was determined using [^{3}H]cholesterol added to the initial samples. For determination of the newly synthesized sterol pattern, the eluate was evaporated to dryness in a vacuum concentrator (Desaga, Heidelberg, Germany), taken up in 50 μL chloroform and applied to silver-ion thin-layer chromatography (SI-TLC) plates which were developed once using a mixture of *n*-heptane/ethyl acetate (2:1, vol/vol) according to Pill *et al.* (23). With [^{14}C]mevalonate, a similar protocol was used.

Determination of cytotoxicity. Cytotoxicity of garlic extracts and inhibitors was determined by means of the leakage of lactate dehydrogenase (LDH) relative to the total activity of LDH after lysing the hepatocytes with 0.1% Triton X-100 as described (17).

Determination of enzyme activities. For the determination of enzyme activities, a liver homogenate (1:10 wt/vol) in 0.1 M potassium phosphate buffer (pH 7.4) containing 4 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid and 2 mM dithiothreitol was prepared with a Dounce homogenizer (Braun Melrungen AG, Melrungen, Germany) from the liver of starved (24 h) rats and diluted 1:8. Either the $10000 \times g$ supernatant or the microsomal fraction was used for the measurements. For testing the garlic extracts, the extracts were added before the dilution step at such a concentration that the proper dilution was reached together with that of the enzyme activities. Enzyme activities were determined as follows: hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) according to Shapiro *et al.* (24) and fatty acid synthase according to Nepokroeff *et al.* (25). Protein was determined following the procedure of Lowry *et al.* (26).

Statistical evaluation. The IC_{50} values (the concentration for half-maximal inhibition) were determined from the dose response curves by use of curve fitting programs on a PC. The data were evaluated statistically using Student's *t*-test. Data are given as means ± SD.

RESULTS

Incubation of primary rat hepatocyte cultures with water-soluble extracts of garlic inhibited the incorporation of [^{14}C]acetate into the sterol fraction extracted from hepatocyte homogenates. Figure 1 illustrates the effect of concentration. Obviously, there is a strong inhibition above 10 mg/mL, although cytotoxic effects were not found below 125 mg/mL within the incubation period of 2 h (Table 1). Between 0.25 and 5 mg/mL, a plateau was observed which was characterized by a mean inhibition of about 23% (Fig. 1). At lower concentrations the inhibition decreased continuously and an IC_{50} value of approximately 90 μg/mL could be determined. In the presence of the well known inhibitor mevastatin, biosynthesis of nonsaponifiable lipids was inhibited by more than 90% at concentrations above 10^{-6} M.

Similar inhibitory effects by garlic extracts were seen with cultures of HepG2 cells. The range of cytotoxic concentrations was about the same (Table 1), while inhibition of sterol biosynthesis was somewhat more sensitive with an IC_{50} value of approximately 35 μg/mL and a maximal inhibition at the plateau phase of about 28% (Fig. 2). At high concentrations, inhibition with HepG2 cells was less pronounced than with rat hepatocytes.

If the radioactively labeled acetate was replaced by labeled mevalonate, the inhibitory effect of the garlic extracts, as well as that of mevastatin largely disappeared in both types of cells (Table 2) indicating that inhibition

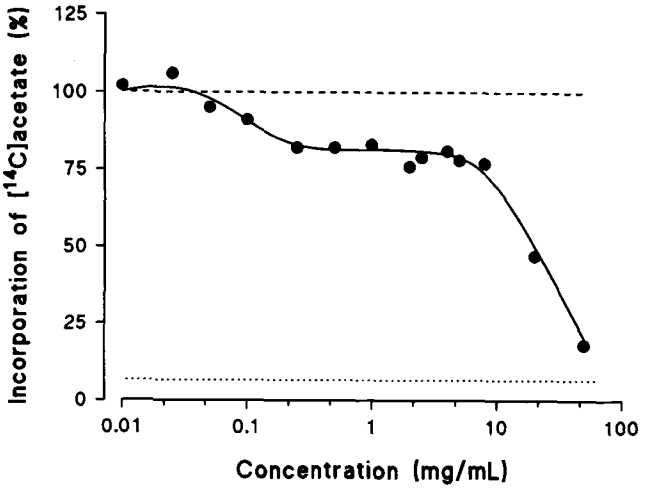

FIG. 1. Dependence of the incorporation of [^{14}C]acetate into nonsaponifiable lipids on the concentration of water-soluble extracts of garlic powder in cultured rat hepatocytes. The dashed line indicates the control level, the dotted line the incorporation in the presence of mevastatin (10^{-6} M). Data represent means ± SD of three to five independent determinations.

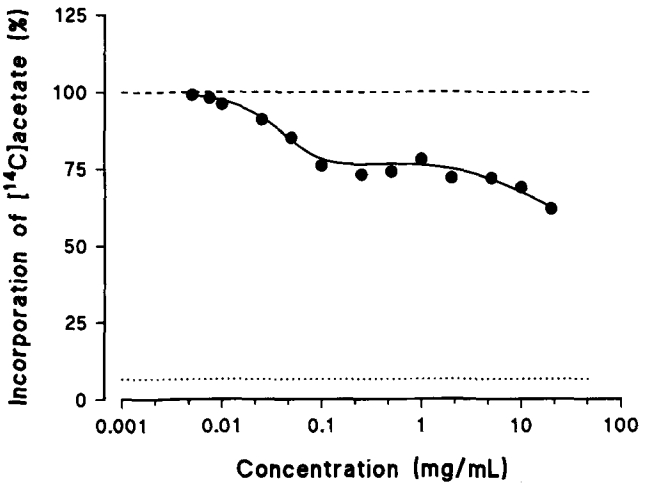

FIG. 2. Dependence of the incorporation of [^{14}C]acetate into nonsaponifiable lipids on the concentration of water-soluble extracts of garlic powder in HepG2 cells. The dashed line indicates the control level, the dotted line the incorporation in the presence of mevastatin (10^{-6} M). Data represent means ± SD of three to four independent determinations.

TABLE 1

Cytotoxicity of Water-Soluble Garlic Extracts on Cultured Rat Hepatocytes and HepG2 Cells

Concentration of garlic extracts (mg/mL)	LDH leakage (% total activity)[a]	
	Rat hepatocytes	HepG2
Control	5 ± 3	2 ± 1
1	5 ± 2	2 ± 1
10	6 ± 1	1 ± 1
50	6 ± 3	3 ± 2
100	12 ± 4	4 ± 2
125	31 ± 7[b]	25 ± 6[b]
150	84 ± 6[b]	67 ± 8[b]
200	100 ± 0[b]	93 ± 6[b]

[a]Data represent means ± SD of triplicate determinations of lactate dehydrogenase (EC 1.1.1.27) (LDH) leakage.
[b]Significant cytotoxicity, $P > 0.001$.

TABLE 2

Inhibition of the Biosynthesis of Nonsaponifiable Lipids from [^{14}C]Acetate or [^{14}C]Mevalonate by Garlic Extracts and Mevastatin in Rat Hepatocytes and HepG2 Cells

Inhibiting material	Inhibition (%)[a]	
	[^{14}C]Acetate	[^{14}C]Mevalonate
Hepatocytes[b]		
Garlic extract[c]	10 ± 3	1 ± 1[d]
Mevastatin[e]	84 ± 7	13 ± 3[f]
HepG2[g]		
Garlic extract[c]	23 ± 6	3 ± 2[f]
Mevastatin[e]	81 ± 8	15 ± 4[f]

[a]Values represent means ± SD of triplicate determinations.
[b]Incorporation of radioactivity for controls was 11.6 ± 1.3 dpm/μg protein and 8.3 ± 1.0 dpm/μg protein for [^{14}C]acetate and [^{14}C]mevalonate, respectively.
[c](100 μg/mL).
[d]Significantly different from acetate: $P < 0.01$.
[e]10^{-5} M.
[f]Significantly different from acetate: $P < 0.001$.
[g]Incorporation of radioactivity for controls was 16.7 ± 2.3 dpm/μg protein and 13.1 ± 1.6 dpm/μg protein for [^{14}C]acetate and [^{14}C]mevalonate, respectively.

occurs at some enzymatic step prior to the formation of mevalonate.

It is known that mevastatin exerts its inhibitory effect on the level of HMG-CoA reductase activity, and preliminary evidence suggested that the same holds true for garlic extracts (17). Therefore, the direct effect of garlic extracts on some enzymes of cholesterol and fatty acid metabolism was tested in detail using liver homogenates from fasted rats. For HMG-CoA reductase, a concentration-dependent inhibition was found starting at concentrations as low as 25 μg/mL (Fig. 3). Above 500 μg/mL inhibition plateaued at about 15% of total enzymatic activity. In the presence of 10^{-5} M mevastatin, almost complete inhibition of the enzyme activity was noted (not shown). Fatty acid synthase was also inhibited (Fig. 3). However, no significant inhibition was found below 100 μg/mL, while maximal inhibition by slightly more than 15% was reached above 1 mg/mL.

The effect of several compounds present in the garlic powder, namely alliin, nicotinic acid and adenosine, on sterol biosynthesis is shown in Figure 4. There was only slight inhibition at high concentrations of alliin (but not at low concentrations, *c.f.* Table 3). Nicotinic acid was shown to be somewhat more potent. Interestingly, adenosine inhibited sterol synthesis to a much greater extent and showed a biphasic concentration dependence similar to that shown by the extracts. After preincubation of alliin with alliinase (from a garlic extract of low concentration), *i.e.* after conversion to allicin (18), a slight inhibition was observed in HepG2 cells only (Table 3) indicating that allicin but not alliin might, under certain conditions, be an active principle. In accordance with this assumption, alliin did not significantly inhibit HMG-CoA reductase

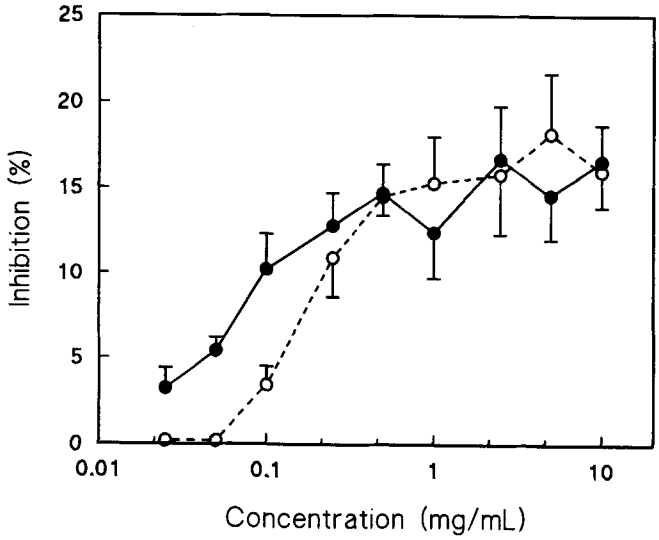

FIG. 3. Dependence of the inhibition of HMG-CoA reductase (● — ●) and fatty acid synthase (O--O) on the concentration of water-soluble garlic extracts. Values represent means ± SD of triplicate determinations.

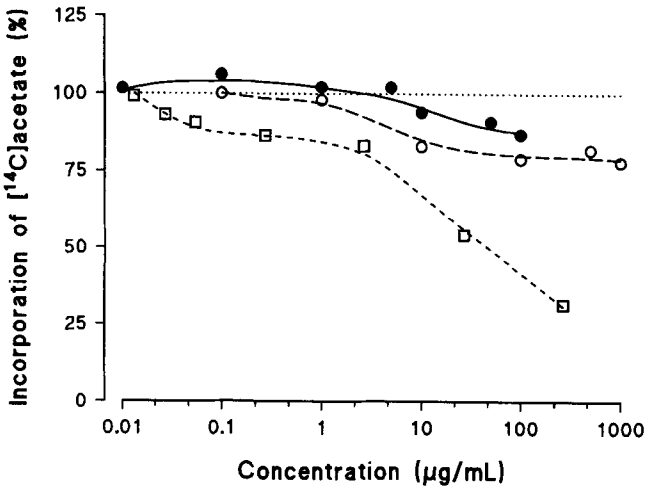

FIG. 4. Dependence of the incorporation of [^{14}C]acetate into nonsaponifiable lipids on the concentration of alliin (● — ●), nicotinic acid (O- - -O) and adenosine (□--□) in cultured rat hepatocytes. The dotted line indicates the control level. Data represent means of duplicate determinations.

in liver homogenates (Fig. 5). Nicotinic acid caused about 10% inhibition above 10 μg/mL. Interestingly, adenosine inhibited HMG-CoA reductase with even greater efficiency (Fig. 5).

When the sterol pattern produced by the rat hepatocytes was analyzed by SI-TLC, cholesterol was shown to be the main sterol formed (Fig. 6, A and B). The precursors lanosterol and 7-dehydrocholesterol amounted to 8 and 4%, respectively. In the presence of garlic extracts at concentrations above 0.5 mg/mL, a shift in the proportions of the sterols could be observed (Fig. 6A). Lanosterol and, to some extent, 7-dehydrocholesterol, increased at the expense of cholesterol indicating an inhibition at several steps during the conversion of lanosterol to cholesterol. The percentage of 7-dehydrocholesterol never exceeded

TABLE 3

Effect of Alliin with and without Preincubation with Alliinase on the Biosynthesis of Nonsaponifiable Lipids from [^{14}C]Acetate in Rat Hepatocytes and HepG2 Cells

	Incorporation of [^{14}C]acetate into nonsaponifiable lipids[a]			
	Hepatocytes		HepG2	
Compound	dpm/μg Protein	%	dpm/μg Protein	%
None	12.3 ± 1.9	100	15.8 ± 1.1	100
Alliin[b]	11.6 ± 1.3	94	15.0 ± 0.9	95
Alliin (preincubated)[c]	11.4 ± 1.2	93	14.1 ± 0.9[d]	89

[a]Values represent means ± SD of duplicate determinations from four different cultures.
[b]10^{-4} M.
[c]Aliin (10^{-4} M) was preincubated with alliinase from a garlic extract (50 μg/mL) as described in Materials and Methods.
[d]Statistical significance with respective controls: $P < 0.01$.

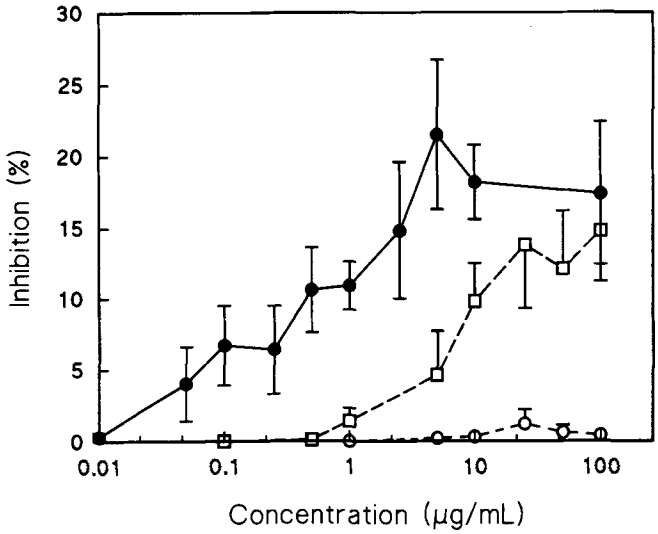

FIG. 5. Dependence of the inhibition of HMG-CoA reductase on the concentration of alliin (O--O), nicotinic acid (□— —□) and adenosine (● — ●). Values represent means ± SD of triplicate determinations.

25% (relative proportion), because at higher concentrations inhibition of lanosterol metabolism predominated. No desmosterol was formed at all concentrations used [an indication to the contrary in a previous abstract (27) was due to inadvertent mislabeling of the respective figure]. With HepG2 cells similar results were obtained (Fig. 6B, Table 4), but pronounced accumulation of lanosterol required even higher concentrations of the extracts than with rat hepatocytes. The proportion of 7-dehydrocholesterol never exceeded 14%. Other (partially unidentified) precursors, presumably dihydrolanosterol (5α-lanost-8-ene-3β-ol), were observed in slightly greater percentages.

Direct addition of several garlic compounds revealed that neither alliin and nicotinic acid nor adenosine (Fig. 7) caused a shift in the composition of the sterol fraction produced by rat hepatocytes. However, when alliin was converted to allicin by preincubation with alliinase, a similar shift in the proportion of cholesterol, lanosterol and

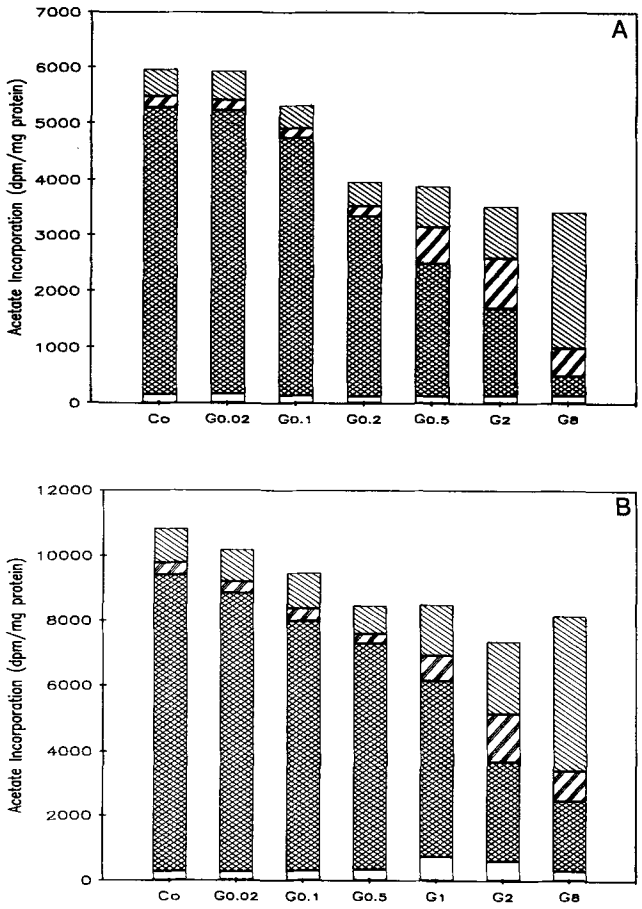

FIG. 6. Influence of different concentrations of garlic extracts on the incorporation of [^{14}C]acetate into cholesterol and cholesterol precursors in (A) rat hepatocytes and (B) HepG2 cells. Co, control; G0.02 to G8, garlic extracts ranging from 0.02 to 8 mg/mL (cross-hatched bars) cholesterol, (left-hatched bars) lanosterol, (striped bars) 7-dehydrocholesterol, (open bars) other sterols.

7-dehydrocholesterol was found as with the garlic extracts (Fig. 7). Similar effects were observed with HepG2 cells (Table 4) or after incubation with mevalonate (not shown). In contrast, mevastatin did not change sterol composition in rat hepatocytes, but caused a considerable reduction of acetate incorporation into the sterol fraction only (Fig. 7). Triparanol and ketoconazole caused marked changes in the sterol composition that were considerably different from those observed with the garlic extracts. With the latter inhibitors, large proportions of desmosterol were formed (Fig. 7).

TABLE 4

Effect of Alliin with and without Preincubation with Alliinase on the Incorporation of [^{14}C]Acetate into Cholesterol and Cholsterol Precursors in HepG2 Cells

Sterol	Incorporation of [^{14}C]acetate into cholesterol and precursor sterols (% of total)[a]		
	Control	Alliin[b]	Alliin (inc.)[c]
Lanosterol	7.7 ± 1.4	9.2 ± 1.6	25.8 ± 2.7[d]
Dihydrolanosterol	0.5 ± 0.3	0.4 ± 0.2	6.1 ± 1.6[d]
7-Dehydrocholesterol	3.9 ± 0.8	5.9 ± 1.5	10.7 ± 1.7[d]
Cholesterol	87.1 ± 5.3	83.7 ± 7.0	55.9 ± 3.8[d]
Others	0.8 ± 0.4	0.8 ± 0.5	11.5 ± 2.1[d]

[a]Values are expressed as percentage of radioactivity in relation to the total incorporation in each group and represent means ± SD from three to four experiments.
[b]10^{-4} M.
[c]Alliin (10^{-4} M) was preincubated (inc.) with alliinase from a garlic extract (50 μg/mL) as described in Materials and Methods.
[d]Statistical significance with control: $P < 0.01$.

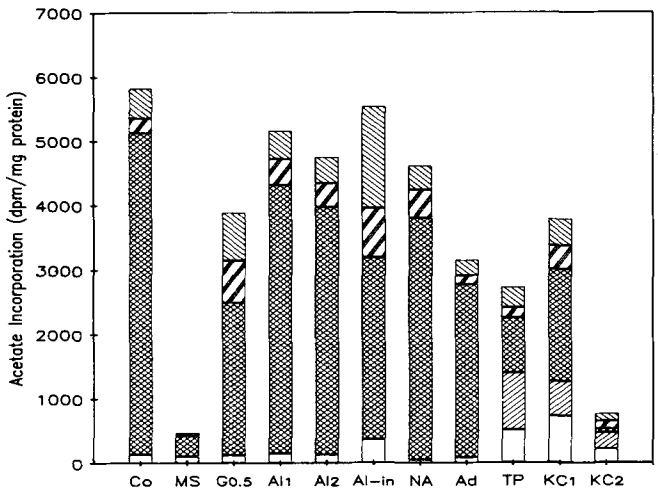

FIG. 7. Influence of different inhibitors and garlic compounds on the incorporation of [^{14}C]acetate into cholesterol and cholesterol precursors in rat hepatocytes. Co, control; MS, mevastatin; G0.5, garlic extract (0.5 mg/mL); AL1, alliin (10^{-4} M); AL2, alliin (10^{-3} M); AL-in, alliin (10^{-3} M) preincubated with alliinase; NA, nicotinic acid (1 mg/mL); Ad, adenosine (20 μg/mL); TP, triparanol (10^{-5} M); KC1, ketoconazole (10^{-5} M); KC2, ketoconazole (10^{-4} M). (cross-hatched bars) Cholesterol, (left-hatched bars) lanosterol, (striped bars) 7-dehydrocholesterol, (right-hatched bars) desmosterol, (open bars) other sterols.

DISCUSSION

In the present study we report on multiple inhibitory effects of garlic extracts at several different steps (early and late) in the cholesterol biosynthetic pathway. The extent of inhibition depended strongly on the concentration of the extracts.

Firstly, inhibition of total sterol biosynthesis was recognized as a biphasic phenomenon caused mainly on the level of HMG-CoA reductase. The fact that rat hepatocytes and human HepG2 cells responded very similarly may indicate that the results are relevant for humans consuming fresh garlic or equally potent garlic preparations. Indeed, the degree of inhibition observed at low concentrations (25-30%) nicely reflects the decrease in cholesterol levels described for rat serum (1) as well as human serum (4,5). The high concentrations studied herein, which cause much stronger inhibition comparable to that by mevastatin, are not likely to be reached even during excessive consumption of garlic preparations.

Studies in which acetate was replaced by mevalonate and direct measurement of enzyme activities suggested that the garlic extracts exert their lowering effect essen-

tially through inhibition of HMG-CoA reductase. A similar inhibition with water-soluble extracts had previously been observed with the chicken liver enzyme (28). Which compounds contained in the extracts are responsible for this inhibition is not known. As shown in this study, nicotinic acid and adenosine may be among the candidates. The relative concentration of nicotinic acid in garlic extracts (approximately 4 μg/mL, estimated from Ref. 29), however, seems too low to account for the total inhibition observed with the garlic extracts. Adenosine, on the other hand, the concentration of which in garlic is higher (approximately 10 μg/mL, estimated from Ref. 29) seems to contribute to the inhibition of cholesterol biosynthesis in an indirect manner, since it is unlikely to enter the hepatocytes. Whether garlic extracts raise the intracellular adenosine level remains to be investigated. The contribution of alliin and its product allicin to the inhibition at the level of HMG-CoA reductase appears of minor importance, although the effect of allicin in HepG2 cells was significant. Thus, it seems most likely that other still unknown components may also be involved in the inhibition. Hepatocyte cultures may aid in further defining these active principles and their mode of action.

Secondly, exposure of rat hepatocytes or HepG2 cells to higher concentrations of garlic extracts resulted in a pronounced shift in the composition of the sterol fraction indicating inhibition at later stages of cholesterol biosynthesis. This inhibition led to the pronounced accumulation of lanosterol and, to a lesser extent, of 7-dehydrocholesterol. The latter compound represents 6-9% of total sterols in subcellular membranes of normal rats (30). The accumulation of lanosterol is comparable to the effect of ketoconazole, a known antimycotic, and of buthiobate which both have been found to inhibit the conversion of lanosterol to demethyllanosterol (14,15,31). Whether the same enzymatic step is inhibited by the garlic extracts remains to be investigated. On the other hand, the accumulation of 7-dehydrocholesterol seems much less than with other inhibitors such as *trans*-1,4-*bis*(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (32) and 4-(2-[1-(4-chlorocinnamyl)piperazin4-yl]ethyl)benzoic acid (10). This indicates that inhibition of 14α-demethylation by the garlic extracts might be much more pronounced than that of side-chain saturation. Desmosterol, which is known to cause adverse effects as a consequence of massive accumulation, was not formed in the presence of garlic extracts. Thus, side-chain saturation again appears less inhibited. In contrast, triparanol in our hepatocyte cultures gave rise to desmosterol which is in agreement with the known accumulation of desmosterol *in vivo*, particularly in the skin (33,34), as well as *in vitro* (15). Although the interference of garlic compounds with late stages of cholesterol biosynthesis is very interesting from the molecular point of view, it seems to be of minor therapeutic significance, because it requires concentrations one or two orders of magnitude above those affecting the early stage. These concentrations seem too high to be reached during normal and even long-term consumption of garlic preparations.

Neither nicotinic acid nor adenosine seem to be involved in the inhibition at late stages of cholesterol biosynthesis. However, there is some indication that allicin at high concentrations might be involved in this inhibition in both types of cells. Whether this effect is specific for this compound or is observed also with other garlic compounds derived from alliin, which contain sulfur- and allyl-groups, is currently being investigated.

It is concluded that defined compounds present in water-soluble extracts from garlic inhibit the biosynthesis of cholesterol in hepatocytes thus contributing to the reduction of serum cholesterol. The observed gap between the low concentrations inhibiting the early steps and the high concentrations required to inhibit the late steps in the synthetic pathway may aid in lowering cholesterol biosynthesis without accumulating undesirable cholesterol precursors.

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The Esterification of Cholesterol in the Yolk Sac Membrane of the Chick Embryo

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The uptake of lipid from the yolk by the yolk sac membrane of the chick embryo is accompanied by the rapid esterification of a large proportion of the yolk cholesterol. This could arise from enhanced acyl-CoA:cholesterol acyltransferase (ACAT) activity and/or inhibition of cholesteryl ester hydrolase (CEH) activity. The activity of ACAT was therefore measured in microsomes obtained from yolk sac membranes at various stages of development. A high level of activity (up to 929 pmol of cholesteryl oleate formed per min per mg protein) was found during the second half of this period. Supplementation with exogenous cholesterol stimulated ACAT activity in microsomes obtained from the tissue at the earlier, but not at the later, stages of development suggesting that the enzyme became saturated with microsomal cholesterol as development proceeded. Correlating with this, the concentration of cholesterol in the microsomes increased 4-fold between 9 and 20 d of development. The activity of CEH was very low in the microsomes and could not be detected in the cytosolic fraction. The activity of a protein, which has been shown to function as an inhibitor of CEH, was found to be present at all stages of development. The high activity of ACAT, together with the low activity of CEH and an active CEH inhibitor protein is a combination well suited to promote an essentially unidirectional conversion of cholesterol to cholesteryl ester. This process may be a major determinant of the rate of lipid transfer from the yolk to the embryo. *Lipids 28,* 621-625 (1993).

The importance of yolk lipids as a source of energy, membrane components and essential fatty acids for the developing chick embryo is well recognized (1). During the early stages of development, the yolk becomes completely surrounded by the yolk sac membrane, a structure which is physically an extension of the embryo's hind-gut (1). This membrane mediates the transfer of nutrients from the yolk to the body of the embryo by absorbing lipids from the yolk and converting them into lipoproteins which are released into the embryonic circulation. In the yolk, cholesterol exists predominantly as the free sterol, but it is extensively (80%) esterified in the yolk sac membrane (1-3). This rapid conversion of yolk cholesterol into cholesteryl esters in the endodermal cells has led to the suggestion that the esterification of cholesterol is important for the formation and stability of the lipoproteins released into the circulation, and may be an important factor in determining the rate of lipid transfer from yolk to embryo (1-3).

Tissues which are very active in cholesterol metabolism, such as the liver and adrenal cortex, often have a high level of acyl-CoA:cholesterol acyltransferase (ACAT) (4,5). Furthermore, such tissues also contain appreciable levels of neutral cholesteryl ester hydrolase (CEH) activity (6-11) which reverse the effects of ACAT action. Thus the net rate of conversion of cholesterol to cholesteryl esters in the yolk sac membrane may be determined by the relative activities of ACAT and CEH at the appropriate subcellular sites in the endodermal cells.

The transfer of lipid to the chick embryo is particularly intensive during the final week of development (1), and this together with the high cholesteryl ester content suggests that the endodermal layer of the yolk sac membrane may contain a high level of ACAT linked to a more moderate CEH activity. To investigate this possibility, we have measured the activities of ACAT and of CEH in the relevant subcellular fractions of yolk sac membranes obtained from embryos at various stages of development. In addition, the activity of a cytosolic protein, which we have recently identified (12,13) and which apparently functions as a physiological inhibitor of CEH, was also measured. Consistent with the role of the yolk sac membrane in converting yolk cholesterol to cholesteryl esters as part of the intensive lipid transfer process, the activities of the relevant enzymic systems in this tissue were found to be very strongly directed toward esterification, with very little propensity for cholesteryl ester hydrolysis.

Some aspects of this work have been previously published in the form of a conference abstract (14).

MATERIALS AND METHODS

Embryos. Fertile eggs from broiler-breeder hens were obtained from commercial poultry suppliers and incubated at 37.8°C and 60% relative humidity in a forced-draught incubator with automatic egg turning. Three separate experiments were performed, and at each developmental stage studied, four replicate groups, each consisting of four yolk sac membranes, were washed free of yolk in ice-cold 0.88% (wt/vol) NaCl and were rapidly frozen in liquid nitrogen. These samples were subsequently used for the determination of the activities of ACAT, CEH and the CEH-inhibitor protein, and for the measurement of microsomal cholesterol and cholesteryl ester levels. Additional samples of yolk sac membrane and yolk were collected, stored at −20°C and subsequently used for the analysis of whole tissue lipid composition.

Lipid analysis. Samples of yolk and of intact yolk sac membrane were homogenized in a suitable excess of chloroform/methanol (2:1, vol/vol), and total lipid extracts were prepared (15). Lipids were separated into their major classes by thin-layer chromatography on silica gel G (Merck, Darmstadt, Germany) using a solvent system of hexane/diethyl ether/formic acid (80:20:1, by vol). Following transmethylation (15), the fatty acid composition of the major lipid classes was determined by gas-liquid chromatography on a packed column of 15% CP Sil 84 on Chromosorb WHP (Chrompak, Middleburg, The Netherlands) in a Packard (Middleburg, The Netherlands) model 428 Gas Chromatograph. The relative proportions of the fatty acids present and the amount of lipid associated with each lipid class were quantified with a

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase; MES, 2-(*N*-morpholino)ethanesulfonic acid; TRIS, *tris*(hydroxymethyl)aminomethane; VLDL, very low density lipoprotein.

Spectra-Physics SP4270 Integrator (Spectra Physics, St. Albans, United Kingdom).

Tissue fractionation. A homogenate was prepared from washed, frozen yolk sac membranes, using the method described for liver by Shand and West (12), and centrifuged at 10,000 × *g* for 15 min. The resulting supernatant was then centrifuged at 100,000 × *g* for 30 min, and the clear cytosol below the floating fat layer was retained. The 100,000 × *g* microsomal pellet was washed in fresh homogenization buffer, centrifuged for a further 30 min at 100,000 × *g* and finally resuspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 50 mM *tris*-(hydroxymethyl)aminomethane (TRIS)/HCl buffer pH 7.2. Aliquots of the cytosols were converted into acetone/diethyl ether powders as already described (12). Further samples of cytosol were delipidized by treatment with the lipophilic Sephadex derivative, hydroxyalkoxypropyl dextran (Sigma, Poole, United Kingdom) (16) for 10 min at 0°C at a concentration of 2% (wt/vol). The lipophilic dextran was removed by centrifugation and the process repeated once.

Enzyme assays. ACAT was assayed with and without the addition of exogenous cholesterol (250 μg/mL) as a suspension in Triton WR-1339 as previously described (17) except that the incubation time was limited to 5 min to ensure linearity. Neutral CEH was measured in microsomes and cytosol essentially according to Shand and West (12) using 50 μg protein incubated for 45 min with high specific activity substrate (cholesteryl [9,10-^{3}H]oleate, 1.6 × 10^6 dpm, 20 nmol). Cytosols were assayed for CEH in the presence of 0.2% sodium taurocholate before and after delipidation.

Assay of the CEH inhibitor protein. Acetone/diethyl ether powders of the yolk sac membrane cytosols were extracted with 50 mM MES, 50 mM TRIS/HCl buffer pH 7.2 and the extracts assayed for inhibitory activity against the rat mammary microsomal CEH by a modification of the method described by Shand and West (12). Briefly, an ethanolic solution of cholesteryl [9,10-^{3}H]oleate (250,000 dpm, 20 nmol) was added to a suspension of the inhibitory protein (0.2–0.4 μg) in the extraction buffer. After preincubation at 37°C for 5 min, the reaction was started by the addition of a solution of bovine serum albumin (0.2 mg) containing the mammary microsomal suspension (100 μg). After a 20-min incubation period, the reaction was stopped and the extent of ester hydrolysis determined.

Estimation of microsomal cholesterol and cholesteryl esters. Lipids were extracted (18) from the microsomal suspensions and redissolved in ethanol. Free and total cholesterol were assayed in the ethanolic solution by the fluorimetric method of Gamble *et al.* (19) and the esterified cholesterol estimated by difference.

Protein measurement. Protein concentrations in microsomal suspensions and in reconstituted cytosolic acetone powders were assayed by the dye-binding method of Bradford (20).

RESULTS

Comparison of the lipid composition of the yolk and yolk sac membrane. The conversion of yolk cholesterol to cholesteryl ester following uptake of lipid by the yolk sac membrane is illustrated in Table 1. Approximately 80% of total yolk cholesterol is present as the unesterified sterol, with only 20% as the ester. In agreement with previous results (1), these proportions are effectively reversed in the yolk sac membrane, where over 80% of the total cholesterol content is present in the esterified form.

TABLE 1

Lipid Composition of Yolk and Yolk Sac Membrane at Day 14 or Development[a]

Lipid class	Yolk	Yolk sac membrane
Cholesterol	5.4 ± 0.2[b]	1.3 ± 0.1
Cholesteryl ester	1.3 ± 0.1[b]	5.6 ± 0.3
Triacylglycerol	67.4 ± 0.4	67.8 ± 1.2
Phospholipid	25.2 ± 0.3	23.8 ± 1.1
Free fatty acid	0.7 ± 0.1	1.1 ± 0.1
Total lipid content	31.3 ± 0.2	30.8 ± 0.9

[a]Each lipid class is expressed as percentage (wt/wt) of total lipid. Total lipid content is expressed as percentage (wt/wt) of wet tissue. The results are means ± SEM from five separate experiments. Significant differences (Student's *t*-test) between lipid compositions of yolk and yolk sac membrane.
[b]$P < 0.001$.

Activity of ACAT levels of cholesterol and cholesteryl esters in the yolk sac membrane during development. The yolk sac membrane contained high levels of ACAT activity throughout the later developmental period (Table 2). Compared to values reported for various mammalian tissues (17,21–23), the enzyme was already highly active at day 9, but this value was increased nearly 3-fold by day 16. This increase in ACAT correlated with the period of maximal uptake of yolk lipid by the yolk sac membrane (1). The activity of ACAT then decreased (from 929 ± 56 to 592 ± 13 pmol cholesteryl oleate formed per min/mg protein) with the approach of hatching. ACAT activity was increased by the addition of exogenous cholesterol, with the greatest stimulation, approximately 2-fold, occurring at days 9 and 12. Less stimulation was observed at the later stages of development, suggesting that the enzyme in the microsomes may have been approaching saturation

TABLE 2

ACAT Activities in Microsomes Isolated from Yolk Sac Membranes of Embryos During Development[a]

	Cholesteryl oleate formed (pmol/min/mg protein)	
Days of development	Endogenous cholesterol	Exogenous cholesterol
9	326 ± 42[b]	696 ± 72[c]
12	550 ± 71[b]	1100 ± 115
14	720 ± 57[c]	1023 ± 77
16	929 ± 56	1162 ± 49
20	592 ± 13[b]	693 ± 40[c]

[a]Acyl-CoA:cholesterol acyltransferase (ACAT) activities were determined in microsomes obtained from four replicate samples, each consisting of four yolk sac membranes. Exogenous cholesterol was added to the assay mixture as a dispersion in Triton WR1339 to give a final concentration of 250 μg/mL. The data are from one experiment, typical of the three performed, and are the means ± SEM for the four replicate samples. Significant differences from day-16 values were determined by one-way analysis of variance using Fisher's least significant difference test.
[b]$P < 0.001$.
[c]$P < 0.01$.

TABLE 3

Cholesterol and Cholesteryl Ester Content of Yolk Sac Membrane Microsomes[a]

Days of development	Cholesterol (μg/mg microsomal protein)	Cholesteryl ester (μg/mg microsomal protein)
9	26.7 ± 2.5	13.9 ± 1.1
12	33.8 ± 2.0	17.2 ± 1.1
14	37.3 ± 4.1	20.7 ± 1.5
16	60.6 ± 4.8[b]	31.0 ± 2.2[b]
20	102.3 ± 9.8[b]	68.2 ± 7.5[b]

[a]Cholesterol and cholesteryl ester content of microsomes were determined in replicate samples, each derived from four yolk sac membranes. The results are from one experiment, typical of the three performed, and are the means ± SEM of four replicate samples. Significant differences from day-9 values were determined by one-way analysis of variance using Fisher's least significant difference test.
[b]$P < 0.01$.

TABLE 4

The Activities of Microsomal CEH and the CEH-Inhibitor Protein in the Yolk Sac Membrane During Development[a]

Days of development	Microsomal CEH (pmol/min/mg protein)	CEH Inhibitor (% inhibition by 200 ng protein)
9	12.9 ± 1.8	25.8 ± 1.2[b]
12	16.7 ± 1.3	39.3 ± 2.7
14	16.4 ± 0.9	37.1 ± 3.1
16	15.5 ± 1.0	23.6 ± 2.0[b]
20	12.5 ± 0.8	22.4 ± 1.4[b]

[a]The activities of cholesteryl ester hydrolase (CEH) and the CEH-inhibitor protein were determined in the microsomal and cytosolic fractions, respectively, prepared from replicate samples, each consisting of four yolk sac membranes. The results are from one of the three experiments and are the means ± SEM of four replicate samples. Significant differences from day-12 values were determined by one-way analysis of variance employing Fisher's least significant difference procedure.
[b]$P < 0.01$.

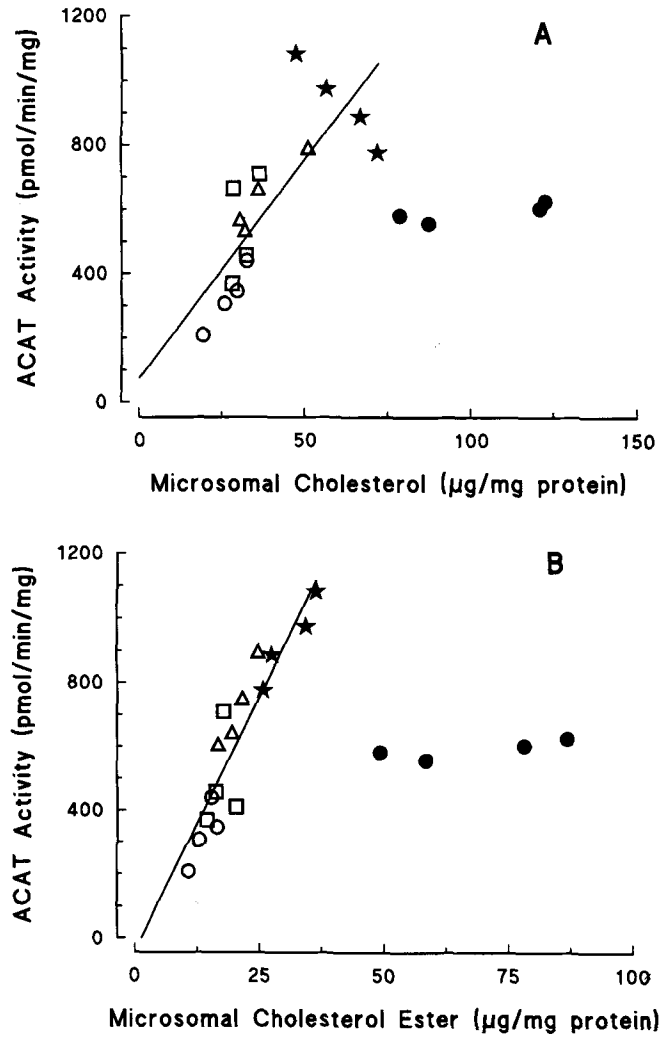

FIG. 1. Scatter diagrams relating acyl-CoA:cholesterol acyltransferase (ACAT) activities, assayed with endogenous cholesterol, to microsomal contents of cholesterol (A) and cholesteryl ester (B). Linear regression analysis was performed on data from day 9 (○), day 12 (□), day 14 (△) and day 16 (★), but data from day 20 (●) were not included. The relationships were linear for both ACAT *vs.* cholesterol (y = 13.63x + 73.33, r = 0.84) and ACAT *vs.* cholesteryl esters (y = 32.89x − 66.45, r = 0.92).

with cholesterol. In support of this concept, the concentration of the endogenous microsomal cholesterol increased almost 4-fold between days 9 and 20 of development (Table 3). A concomitant increase in the microsomal esterified cholesterol was also observed over the same period. ACAT activity was highly correlated with microsomal cholesterol (Fig. 1A) and cholesteryl ester (Fig. 1B) between days 9 and 16 (r = 0.84 and 0.92, respectively), but the correlation coefficient was much lower (r < 0.45) when the day-20 results were included.

Activities of microsomal CEH and the CEH-inhibitor protein in the yolk sac membrane. The microsomal CEH activity was less than 2% of the maximum ACAT activity at each stage studied (Table 4). Moreover, this low enzymic level did not vary significantly over the period of highest yolk lipid mobilization. There was an apparent decrease in activity between days 16 and 20 but this was not significant. Cytosolic CEH activity was undetectable, both before and after delipidation of the cytosols, even when measured in the presence of taurocholate to prevent inhibitor activity (data not shown). In contrast, the cytosolic CEH-inhibitor protein was active throughout the developmental period exhibiting peak activity at day 12 when 200 ng of cytosolic protein caused over 39% inhibition of the mammary microsomal CEH. Inhibitory activity was significantly higher at days 12 and 14 than at the beginning and end of the development period studied (Table 4).

Fatty acid composition of the cholesteryl esters of the yolk sac membrane. Previous studies have established that cholesteryl oleate is the major species of esterified cholesterol synthesized in the yolk sac membrane (1). The data presented in Table 5 show that the proportion of oleic acid increased from approximately 48% at days 9 and 10 to approximately 77% at day 20, indicating that the fatty acid profile of the cholesteryl esters was altered during development.

DISCUSSION

During the final week of chick embryo development, lipid is transferred from the yolk at an average rate of over 500

TABLE 5

Fatty Acid Composition of Cholesteryl Ester in the Yolk Sac Membrane[a]

Fatty acid	Percentage (wt/wt) of total fatty acids		
	Day 9,10[b]	Day 14	Day 20
16:0	11.7 ± 2.2	6.2 ± 0.6	3.3 ± 0.1[c]
16:1	1.6 ± 0.2	1.4 ± 0.4	1.2 ± 0.1
18:0	6.8 ± 1.3	4.7 ± 0.1	3.9 ± 0.1
18:1	47.7 ± 3.6	66.1 ± 1.5[c]	76.9 ± 0.5[d]
18:2	18.6 ± 2.4	17.1 ± 1.8	12.3 ± 0.3
18:3	3.1 ± 2.3	1.5 ± 0.2	1.2 ± 0.1
20:4	2.9 ± 0.1	1.6 ± 0.2[c]	0.6 ± 0.2
22:6	6.1 ± 0.4	n.d.[e]	0.7 ± 0.3[f]

[a]The fatty acid composition of the cholesteryl ester isolated from yolk sac membrane was determined as described in the Materials and Methods section. Only the major fatty acids are shown. The results are means ± SEM of three separate experiments. Significant differences were determined by Student's *t*-test and show differences from day-9,10 values.
[b]In two of the experiments, samples were taken at day 9, whereas in the third experiment the sample was taken at day 10.
[c]$P < 0.05$.
[d]$P < 0.01$.
[e]n.d. = not detectable.
[f]$P < 0.001$.

mg per day (1). Over this period the endodermal cells of the yolk sac membrane are highly active in vesicular translocation events involving phagocytosis of yolk contents at the apical surface and the subsequent exocytosis of lipoprotein particles at the basal surface (1,24). The evidence that triacylglycerols and phospholipids are subject to extensive hydrolysis and re-esterification following uptake (1,3) implies that this tissue is also highly active in the biochemical processes involved in the reassembly of lipids and their packaging into lipoprotein particles. In addition, previous work has shown that, within the endodermal cells, yolk lipid is subjected to certain biochemical transformations involving fatty acid desaturation and elongation (1,2). However the most dramatic change in lipid metabolism which occurs during translocation across the endodermal layer is the conversion of the major portion of yolk cholesterol to cholesteryl ester (Refs. 1,2 and Table 1). The present study has demonstrated a corresponding increase in ACAT activity in the yolk sac membrane over the same period.

Measurement of microsomal ACAT activity in the presence of endogenous cholesterol reflects the amount of sterol substrate present in the microsomes, whereas maximal enzyme activity can only be measured in the presence of saturating concentrations of substrate, usually supplied exogenously (25). The results obtained under these two conditions (Table 2), considered in association with the changes in cholesterol content of the microsomes (Table 3), indicate that the increase between day 9 and day 12 and the decrease between day 16 and day 20 are due to alterations in the total activity of the enzyme present (Table 2). In contrast, the change in ACAT activity that occurs from day 12 to day 16 is predominantly due to increases in microsomal cholesterol content (Tables 2 and 3). This corresponds to the period during which the uptake of lipid by the tissue is most rapid. Taken together, the results indicate that the potential for cholesterol esterification in the yolk sac membrane increases between days 9 and 16, due both to an elevation in the activity of ACAT and to the increasing supply of substrate to the enzyme. The decrease in ACAT activity at day 20 occurs despite the presence of abundant endogenous cholesterol. This is reflected in the poor correlation shown between ACAT activity and microsomal cholesterol content at this point (Fig. 1A). Although the reason for this is not clear at present, it is unlikely to be due to inhibition by the large amount (68.2 ± 7.5 μg/mg microsomal protein) of cholesteryl ester present, since addition of cholesteryl oleate (100 μg/mg microsomal protein) gave no evidence of inhibition (data not shown).

The activities of ACAT, reported here for the yolk sac membrane (Table 2) are high in comparison with previously published values for various mammalian tissues. For example, the activities reported for tissues which are not regarded as major sites of cholesterol esterification, such as the mammary gland, heart, brain and pancreas, are generally less than 1% of the activity observed in the yolk sac membrane at day 16 (17,26,27). Even tissues which are actively involved in cholesterol metabolism, such as liver, intestine, ovary and adrenal cortex, typically express levels of ACAT which are less than 20% of those reported here for the yolk sac membrane (21–23,25,28,29). However, in this latter group of tissues, conditions which increase the requirement for cholesterol esterification, such as the feeding of excess cholesterol (27,28,30) or, in the case of the ovary, luteinization (31), elevate ACAT activity to values approaching those of the yolk sac membrane. It is clear that the levels of ACAT expressed in the yolk sac membrane are similar to the highest values so far reported in other systems. In contrast to ACAT, the activity of microsomal CEH was low throughout the developmental period (Table 4). This activity would have been further diminished *in vivo* by the CEH-inhibitory activity present at all of the stages studied (Table 4). Cytosolic CEH activity could not be detected nor was there any evidence for translocation of the putative cytosolic CEH to the "fat cake," as has been reported for hormone sensitive lipase in adipocytes (32), since no activity could be detected either before or after delipidation. Thus, the capacity to hydrolyze cholesteryl esters formed by ACAT was extremely low, suggesting that the relevant enzyme systems in the yolk sac membrane were very strongly directed to the net esterification of incoming cholesterol.

Cholesterol oleate is the major species of cholesteryl ester produced in the yolk sac membrane (1,2), and high levels of this ester also accumulate in the embryo liver (1–3). It is nevertheless clear that the total amount of cholesteryl oleate in the yolk sac membrane increases throughout development. This may reflect either an increase in the specificity of ACAT for oleoyl-CoA, arising from covalent modifications or noncovalent interactions of the enzyme or, more likely, an increased availability of oleic acid due to the action of Δ9 desaturase in this tissue (1,2).

The high proportion of unesterified cholesterol that is found in the yolk is a direct consequence of the mechanism of yolk formation in the laying hen, as discussed by Griffin (33). Yolk lipid is derived from very low density lipoprotein (VLDL) particles secreted by the liver of the laying hen which are not only much smaller than those found

in the immature bird but also have a different apoprotein profile so that they are directed specifically to the ovarian follicle (33). These unusually small VLDL particles have a high surface-to-volume ratio and, since cholesterol is a surface component of such particles while cholesteryl esters are located in the core, this results in a high proportion of cholesterol to cholesteryl esters in the lipoproteins deposited in the ovum. Excess cholesterol is deleterious to membrane function, and cells usually maintain a remarkably constant level of free cholesterol, storing the excess in the esterified form. The influx of the large amount of yolk cholesterol into the endodermal cells of the yolk sac membrane could therefore be potentially damaging if the cellular metabolism could not esterify the excess sterol. It is clear, from the results presented here, that the endodermal cells do adapt by enhancing their effective ACAT activity during the period of most rapid cholesterol transfer. This could arise either from activation of existing enzyme molecules or by an increase in the amount of enzyme protein, although evidence to decide between these two possibilities is not currently available.

Apart from the function of storage, there is also more recent evidence that the production of cholesteryl esters may be an obligatory stage in the assembly of stable lipoproteins (33,34). Cholesteryl esters are synthesized by ACAT at the cytoplasmic face of the endoplasmic reticulum. They are extremely hydrophobic and have been shown to accumulate initially between the leaflets of the endoplasmic reticulum bilayer (35). Nascent apoprotein B is also bound co-translationally to the luminal leaflet of the same subcellular organelle, and it has been proposed that it is only released into the cisternal space following interaction with cholesteryl ester (36). A parallel reduction in the secretion of both cholesteryl esters and apoprotein B was observed when hepatic VLDL production was inhibited by lovastatin (33), and this would also support the idea that their secretion was coupled.

Thus, it appears that the assembly of stable lipoprotein particles may be directly linked to the production of cholesteryl esters, suggesting that in the yolk sac membrane cholesterol esterification may be an important determinant of lipid transfer to the embryo. High pre-hatch mortality in eggs from "young parent" chickens may be related to impaired lipid transfer in the embryo (1,2), and investigations into the efficiency of esterification in such embryos are currently in progress.

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Nervonic Acid in Red Blood Cell Sphingomyelin in Premature Infants: An Index of Myelin Maturation?

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The present study addresses the question whether nervonic acid (24:1n-9) accumulation in sphingomyelin (SM) of red blood cells (RBC) could yield information on cerebrum maturation in premature infants. The study included 28 premature eutrophic infants of 31.5 wk gestational age. Eleven were fed with human milk, nine with a regular formula and eight with an α-linolenate-enriched formula. The fatty acid composition of the SM fraction was determined by gas–liquid chromatography on a 50-m fused silica capillary column. At 32 wk gestational age, the main fatty acids in SM were 16:0, 18:0, 20:0, 22:0, 24:0 and 24:1n-9. After five weeks of feeding, at week 37 of postconceptional age, the most striking variation was a rise in 24:1n-9, from 9.9 ± 0.7 to 12.8 ± 0.9 ($P < 0.02$), regardless of regimen in all three feeding groups. The rise in 24:1n-9 after birth in premature eutrophic infants is the beginning of a trend toward the higher levels in 24:1n-9 observed in mature newborns and older infants. The 24:1n-9 level in SM of RBC from premature infants may reflect 24:1n-9 levels in SM of brain and could thus reflect brain maturity.
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In recent years, the need for n-3 polyunsaturated fatty acids (PUFA) in early brain development has been pointed out by many investigators (1–6). However, few studies have been devoted to the metabolism and the role of nervonic acid (24:1n-9) which, together with lignoceric acid, constitutes the major long chain fatty acid of myelin. Accumulation of these two acids may reflect the increase in myelin during gestation in animals (7) and in humans (8). Nervonic acid, which is synthesized by elongation of oleic acid (9), is also characteristic of the sphingomyelin (SM) of red blood cells (RBC) and is one of the four major fatty acids of this lipid fraction in adults (10,11) and in infants (12).

The only data on nervonic acid distribution in infant RBC had previously been obtained on full-term newborns (12) during the first few hours after birth (13) and during the first month of life (14). The present work was undertaken to investigate whether, in premature infants, nervonic acid might accumulate in SM of RBC as in the cerebrum and thus reflect maturation. It compared three different types of milk-feeding (human, regular formula and α-linolenate-enriched formula) on PUFA metabolism in premature infants. The results suggest that nervonic acid in SM of RBC could indeed be used as an index of maturation in premature newborns.

MATERIALS AND METHODS

The study included 28 premature eutrophic infants (11 males and 17 females) admitted to the Neonatal Department of the Regional Hospital Center of Montpellier for nutritional assessment and treatment. Mean gestational age was 31.5 wk (29 to 33) and mean birthweight 1630 g (1270–1980 g). Eleven infants were fed human milk, nine received regular formula feeding (Pregallia), and eight an α-linolenate-enriched formula (enriched Pregallia). Both formulas were from Nutripharm (Levallois-Perret, France). The α-linolenic acid-enriched formula was obtained by isocaloric partial substitution of fats with colza oil [low-erucic acid rapeseed (LEAR) oil; Codex Alimentarius, Canadian General Standard Board, 1987] from Nutripharm. The fatty acid distribution in percent weight of total diet fatty acids for stearic, oleic, linoleic and α-linolenic acids was 5.05, 25.50, 12.50 and 0.55, respectively, in the regular formula and 5.25, 30.25, 12.50 and 1.95, respectively, in the enriched formula. The percent composition in stearic and oleic acids (the main precursors of nervonic acid) was not very different from that in human milk from the Montpellier lactarium (8.10, 34.90, 12.65, 1.10 for stearic, oleic, linoleic and α-linolenic acids, respectively). Informed consent was obtained from parents and the protocol of the study was approved by the Regional Hospital Center Ethics Committee. Three blood samples were obtained from each of 25 infants at 2 (D_2) and 15 d (D_{15}) of milk-feeding, and the third one (Dterm, the equivalent of full-term) at 37 wk postconceptional age. Two infants discontinued participation in the study before Dterm and one was enrolled after D_2.

Lipid analyses. Blood was collected in Na-heparinized glass tubes. An aliquot was taken for hematocrit measurement in order to determine the precise volume of blood corresponding to 200 μL of RBC pelleted under these conditions. The fatty acid composition of SM could thus be expressed as μg of each fatty acid per mL of RBC.

After centrifugation (2000 rpm for 10 min) the RBC pellet was washed by resuspending and centrifugation in isotonic saline solution containing 1 mM ethylenediaminetetraacetic acid (EDTA). The RBC lipids were extracted according to the method of Folch *et al.* (15) using a chloroform/methanol mixture containing 1 mM butylated hydroxytoluene (BHT) (Sigma Chimie, St-Quentin-Fallavier, France) as an antioxidant. Chloroform, pure analytical grade, was from SDS (Peypin France) and methanol from Prolabo (Paris, France). The extract was left to evaporate until dry under a nitrogen stream and the residue was redissolved in a minimal quantity of chloroform/methanol mixture (1:1, vol/vol) for thin-layer chromatography on Silica gel 60 F_{254} (Merck, Darmstadt, Germany) using chloroform/methanol/H_2O (65:25:4, by vol) as developing solvent (16), to which BHT (1 mM) was added.

The phospholipid fractions were visualized at 250 nm after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol (Merck). SM was identified by comparison to known standard (Sigma Chimie).

The silica gel bands containing SM were scraped off and 7.5 μg (125 μL of a methanol solution) of free heneicosanoic acid 21:0 (Sigma Chimie) was added as an internal

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Abbreviations: BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; LEAR oil, low-erucic acid rapeseed oil; PUFA, polyunsaturated fatty acids; RBC, red blood cells; SM, sphingomyelin.

standard. The fatty acids were transesterified with sulfuric acid in methanol (1:19, vol/vol) at reflux. The methyl esters were extracted with hexane and analyzed by gas-liquid chromatography (17) with the following modification: a fused silica capillary column (50 m × 0.32 mm) CP Sil 88 from Chrompack (Les Ullis, France) was used on a Fractovap 2900 Chromatograph (Erba Science, Massy, France). The temperatures were: ionization detector, 250°C; inlet, 230°C; oven program, 10°C/min from 100 to 170°C and 2°C/min from 170 to 200°C.

The amount of each fatty acid was calculated by reference to the internal standard of 21:0 using an ENICA-21 integrator (Delsi France, Argenteuil, France). Results are expressed ± SEM, and Student's paired *t*-test was used for statistical analysis.

RESULTS

The fatty acid composition of SM of premature infant RBCs is reported in Table 1. Results are expressed both as a percent distribution of individual fatty acids and as μg per mL of RBC. The main variation over time concerns nervonic acid (24:1n-9)—a significant rise is observed from 9.9 ± 0.7 to 12.8 ± 0.9 percent between D_2 and Dterm ($P < 0.02$), which is compensated for by small declines in percentages of 16:0 and 18:0. Similar observations can be made when the results are expressed as μg of each fatty acid per mL of RBC. Whereas the total amount of SM fatty acid decreased between D_2 and Dterm, the mean concentration of nervonic acid rose from 29.8 ± 3.6 to 34.4 ± 3.4, reflecting an increased contribution of this acid to the total fatty acid content of SM in RBC during this period.

Figure 1 shows the time course of the variations in percent nervonic acid in the SM of the RBC of each infant. There is an overlap zone ranging from 5 to 15% between values at D_2 and Dterm, but about 80% of the infants

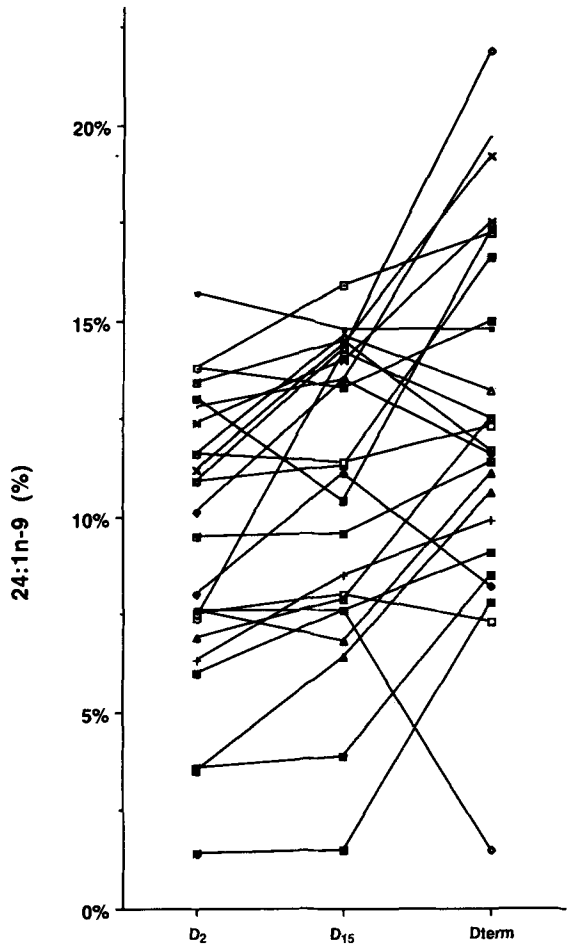

FIG. 1. Time course of the variation in percent nervonic acid in sphingomyelin of red blood cells from 28 premature infants between birth (wk 32 of gestation) and the wk 37 postconceptional age. Abbreviations as in Table 1.

TABLE 1

Variation in the Fatty Acid Composition of Red Blood Cell Sphingomyelins in Premature Infants Between Birth (wk 32 of gestation) and Theoretical Full-Term[a]

	Fatty acid composition (%)			Fatty acid composition (μg/mL of RBC)		
	D_2[b] n = 27[c]	D_{15} n = 28	Dterm[b] n = 26	D_2[b] n = 27	D_{15} n = 28	Dterm[b] n = 26
16:0	37.3 ± 1.3	36.7 ± 1.2	35.3 ± 1.4	97.4 ± 5.1	90.4 ± 5.1	88.8 ± 5.0
18:0	17.8 ± 0.6	16.6 ± 0.6	15.7 ± 0.7[d]	49.0 ± 3.9	41.9 ± 3.0	41.6 ± 4.1
20:0	2.5 ± 0.1	2.7 ± 0.1	2.7 ± 0.1	6.7 ± 0.4	6.7 ± 0.4	6.7 ± 0.4
22:0	6.0 ± 0.2	6.5 ± 0.2	6.6 ± 0.2	16.6 ± 1.2	16.3 ± 1.1	17.0 ± 1.1
24:0	15.3 ± 0.9	15.6 ± 0.8	15.2 ± 1.0	44.8 ± 4.6	40.6 ± 3.3	39.5 ± 3.5
24:1n-9	9.9 ± 0.7	11.0 ± 0.7	12.8 ± 0.9[e]	29.8 ± 3.6	29.0 ± 2.7	34.4 ± 3.4
18:1n-9	2.0 ± 0.3	1.8 ± 0.2	2.0 ± 0.3	6.1 ± 1.0	4.8 ± 0.7	5.7 ± 1.1
18:2n-6	0.4 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	1.4 ± 0.3	1.1 ± 0.2	1.3 ± 0.3
18:3n-3	trace	trace	trace	trace	0.1 ± 0.0	0.1 ± 0.0
20:4n-6	0.4 ± 0.1	0.3 ± 0.0	0.5 ± 0.1	1.1 ± 0.2	0.7 ± 0.1	1.7 ± 0.6
20:5n-3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
22:6n-3	trace	trace	trace	trace	trace	trace
Total	91.8	91.7	91.4	253.1	232.0	237.0

[a]Mean ± SEM of sphingomyelin fatty acids. RBC, red blood cells; D_2, day 2 of milk feeding; D_{15}, day 15 of milk feeding; Dterm, week 37 of postconceptional age.
[b]Student's *t*-test (D_2 *vs.* Dterm).
[c]n = Number of patients.
[d]$P < 0.05$.
[e]$P < 0.02$.

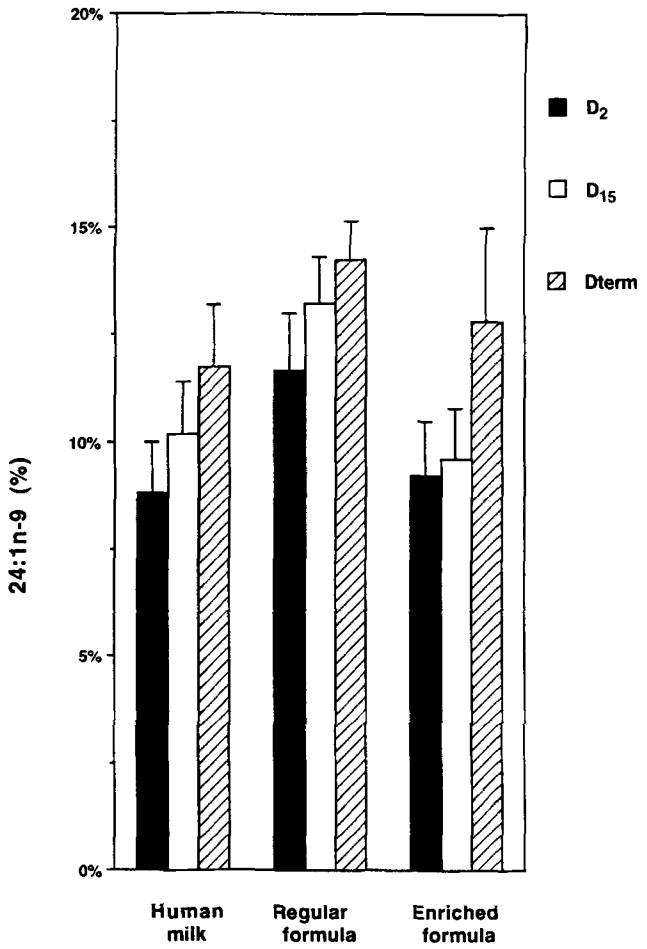

FIG. 2. Time course of the variation in percent nervonic acid in sphingomyelin of red blood cells from three groups of premature infants fed human milk, regular formula and α-linolenate-enriched formula. D_2 and D_{15} correspond to 2 and 15 d of feeding and Dterm to 37 wk postconceptional age.

experienced an increase. In two patients, the pattern was atypical. In the first case, the level, which was 7% on D_2 and D_{15}, drastically declined to 1% at Dterm. In the second case, where the mother had undergone treatment for a pre-eclamptic syndrome, the level rose from 1% (D_2-D_{15}) to 7% (Dterm).

In Figure 2, the infants are considered as a function of their feeding (human milk, regular formula feeding or α-linolenic colza oil-enriched formula feeding). Mean percent nervonic acid increased progressively between D_2 and Dterm in the three groups, suggesting that this increase is independent of the type of feeding (milk or formula feeding).

DISCUSSION

A comparison of the fatty acid composition of the brain of precocial and nonprecocial species has revealed considerable variations in the proportions of nervonic acid around full-term (18). In the guinea pig (precocial species), the main rise (fivefold) in nervonic acid is observed during the 25 d before birth, whereas in the rat (nonprecocial species) it occurs between birth and adulthood (fourfold). In humans, born at term, brain development is accompanied by an approximately 10% increase (15.1-26.4%) in total brain cerebrosides nervonic acid levels and a 15% increase (13.0-28.0%) in sulfatides that occur between birth and two months. Maximum levels are being reached at four years (about 43%) and then remain almost constant until and throughout adulthood (19). This accumulation in brain nervonic acid is related both to an increase in the relative abundance of myelin *vs.* gray matter and to a specific increase in the actual percentage of nervonic acid in myelin SM (10-32% between 1 and 6 yr) (20). The relationships and exchanges between nervonic acid pools in RBC and the central nervous system are not well understood, but published data indicate a rise in nervonic acid in the SM of RBC between birth at term (11.2% at 7 d) (14) and the fifth year of life (25.4%) (21).

The results of the above study indicate that nervonic acid accumulation in SM of RBC starts earlier and that the rise we observe between a postconceptional age of 32-37 wk (from 9.9 to 12.8%) is part of a process extending over a longer period of time and beginning much earlier than full-term. It is to be compared to the accumulation in the cerebrosides observed in the cerebrum of human fetuses obtained after medical termination of pregnancy at this time (1.9-10% of fatty acids between 30-32 wk and full-term) (8). Apparently, nervonic acid levels increase simultaneously in the SM of RBC and in the central nervous system from at least six weeks before full term until the fourth year. This parallelism is surprising as very long chain fatty acids are derived from two different pools—from the circulation and hepatic synthesis for RBC and mainly from *"in situ"* synthesis in the brain. Simultaneous accumulation in two different compartments could imply, amongst other factors, exchanges between the pools or simultaneous maturation of the biosynthetic systems. This needs to be investigated further.

With regard to nervonic acid, the SM of RBC could be an indication of the fatty acid composition of tissue membranes, and especially of myelin cerebrum membranes, and thus nervonic acid could be considered as an index of myelin maturation. Maternal dietary restriction was recently shown to result in a drastic decrease of 24:0 and 24:1n-9 in cerebrosides and sulfatides of suckling rats (7). Whether the levels of 24:1n-9 in SM of RBC could provide information on the consequences of undernourishment in the myelination process of the central nervous system remains to be established. We have recently observed that the levels of stearic and oleic acids are significantly higher in fetal than in maternal plasma lipids between week 18 and week 37 of gestation (22), suggesting that these two acids are of special importance to the fetus, possibly in supplying C_{24} fatty acids. Moreover, in the same study, the oleic acid level decreased by about 25-30% in fetal plasma. Such a decrease at a time of high energy requirements when nervonic acid biosynthesis is particularly active could increase the demand for oleic acid.

In the present study, the premature infants received three different types of feeding (human milk, regular formula and α-linolenic acid-enriched formula). An increase in nervonic acid was observed in all three groups, indicating that endogenous synthesis together with milk or formula feeding provided enough precursors for the

normal evolution of nervonic acid in the SM of RBC and probably in the central nervous system; the percentages of oleic and stearic acids in formula and milk feeding were about 25-35 and 5-8%, respectively. As expected, linolenate supplementation introduced as LEAR oil did not interfere with oleate elongation and conversion to nervonic acid.

A rise in nervonic acid did not occur in a very small number of the infants, probably as a result of individual variations rather than clinical abnormalities as postconceptional ages and birthweights were within the same limited range. One infant, whose mother had pre-eclamptic syndrome and was being treated with glucocorticoids, antimineralocorticoids and a β-blocker, had very low levels of nervonic acid (about 1%) at birth. This might have been due to the medical treatment of the mother, to an alteration in the hemodynamic conditions of the feto-maternal unit or to other factors, such as the specific gestational conditions, because a tendency to recovery occurred several weeks after birth, although this recovery only reached to the lower end of the overall range for the group.

Overall, our results suggest that the incorporation of nervonic acid into membranes in humans, as reflected by its level in RBC, increases between 32 and 37 wk. Results from the literature show that this evolution proceeds during the first years of development, as values of 25.4% have been reported for five-year-old children (21). A steady state is likely to be reached because the percentage of nervonic acid in SM of RBC remains stable thereafter with values ranging from 21 to 25% having been reported for adults (10,11).

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Phospholipid Molecular Species from Human Placenta Lipids

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The phospholipid molecular species from a large-scale preparation of human placenta lipids were analyzed. The major placental phospholipids were choline glycerophospholipids (CPL) (53.2 wt%), sphingomyelin (21.7 wt%) and ethanolamine glycerophospholipids (EPL) (14.6 wt%). 1,2-Diacyl-glycerophosphocholine was the most abundant subclass of CPL (91.7 mol%), while EPL contained 1,2-diacyl (54.6 mol%) and 1-alk-1′-enyl-2-acyl (43.8 mol%) subclasses. The level of polyunsaturated fatty acids (PUFA) in total phospholipids was remarkably constant (38.4–39.9 mol%) within all placental batches tested. The long-chain PUFA, mainly 20:4n-6 and 22:6n-3 of the n-6 and n-3 series, respectively, were found in high proportion in all phospholipid classes, especially in EPL (46.7 mol%) and in inositol glycerophospholipids (IPL) (39.9 mol%). CPL and serine glycerophospholipids were much richer in 18:1n-9 and 18:2n-6. High levels of molecular species with arachidonic acid in the *sn*-2 position were found particularly in 1-alk-1′-enyl-2-acyl-glycerophosphoethanolamine (with 24.0 mol% 16:0 and 22.0 mol% 18:0 in *sn*-1 position) and in 1,2-diacyl glycerophosphoinositol with 42.6 mol% 18:0 in *sn*-1 position. EPL subclasses were rich in 22:6n-3, which occurs mainly as 16:0/22:6n-3 (11.7 mol%) in the plasmalogen form and as 18:0/22:6n-3, 16:0/22:6n-3 and 18:1/22:6n-3 in the diacyl forms. Based on their availability and composition, placental phospholipids could be of interest, for example, for supplementing artificial milk preparations with n-3 and n-6 long-chain PUFA for newborn infants with insufficiently developed 18:2n-6 and 18:3n-3 desaturation/elongation.
Lipids 28, 631–636 (1993).

Linoleic (18:2n-6) and linolenic (18:3n-3) acids are precursors of the two essential fatty acid families, *i.e.,* the n-6 and n-3 families, respectively. Linoleic acid is the precursor of arachidonic acid (20:4n-6) and other long-chain n-6 fatty acids (1). Its dietary insufficiency leads to various deficiency symptoms such as dermatitis, liver pathologies, impaired growth and reproductive failure (2,3). The effects of dietary 18:3n-3 deficiency remain unclear, but the acid is assumed to be essential in the nervous system through its desaturated/elongated product, docosahexaenoic acid (22:6n-3) (4,5). Docosahexaenoic acid is essential in the development of neural tissues as its depletion in brain and retina, where it occurs at high levels (6,7), is accompanied by functional defects such as reduced visual acuity and peripheral neuropathy in monkeys (8). Moreover, 80% of brain 22:6n-3 accumulate between 26 and 40 wk of gestation and infants born prior to 32 wk of gestation have low concentrations of brain 22:6n-3 (9). Linolenic acid as the sole dietary source of n-3 fatty acids has been shown to be inadequate for optimal retinal development of very low birth weight neonates (10). A supply of long-chain n-3 polyunsaturated fatty acids (PUFA) was shown to be necessary to sustain retinal rod function similar to that found in very low birth weight neonates fed with human milk, which contains 0.1–0.3% of the total fatty acids as 22:5n-3 and 22:6n-3. Therefore, optimal accretion of 20- and 22-carbon n-6 and n-3 PUFA may depend on an adequate dietary supply, rather than on desaturation and elongation of 18:2n-6 and 18:3n-3. Most of the marketed preterm infant artificial milks are deficient in the long-chain PUFA as compared to human milk (11,12) and could thus be supplemented with PUFA of both the n-6 and n-3 series. In this work, we report that phospholipid molecular species prepared from human placenta are rich in n-6 and n-3 PUFA.

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Abbreviations: BHT, butylated hydroxytoluene; CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids; GLC, gas-liquid chromatography; GPC, glycerophosphocholines; GPE, glycerophosphoethanolamines; GPI, glycerophosphoinositols; GPS, glycerophosphoserines; HPLC, high-performance liquid chromatography; IPL, inositol glycerophospholipids; PUFA, polyunsaturated fatty acids; SPL, serine glycerophospholipids; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Phospholipase C *(Bacillus cereus)* was purchased from Boehringer Mannheim (Mannheim, Germany). Standard phospholipids and diacylglycerols, butylated hydroxytoluene (BHT) and 3,5-dinitrobenzoyl chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel 60G (0.25 and 2 mm) and LK5 plates were provided by Merck (Darmstadt, Germany) and Whatman (Clifton, OH), respectively. Methanol and 2-propanol were from Prolabo (Paris, France). All other solvents were of analytical and high-performance liquid chromatography (HPLC) grade and were purchased from SDS (Peypin, France). The Ultrasphere ODS column (250 × 4.6 mm, 5 μm particle size) was obtained from Beckman (Berkeley, CA).

Preparation of placental lipid batches. Placentae were collected from healthy mothers, carefully examined and selected by the physicians in maternity units and immediately frozen at −20°C until processing. Five different lipid batches (A–E) were prepared. Their countries of origin and their respective wt% were as follows: Batch A: Great Britain, 65; Spain, 16; France, 12; Belgium, 7; Batch B: Thailand, 63; Egypt, 28; Sri Lanka, 9; Batch C: France, 49; Austria, 29; United Sates, 13; Belgium, 6; Thailand, 3; Batch D: Scandinavia, 100; Batch E: Pakistan, 78; Chile, 18; the former Soviet Union, 4. For each preparation, lipids were extracted from 300 kg placenta from full-term human pregnancies. Placentae were chopped and suspended in 300 L of aqueous solution containing 7.5 g/L NaCl and 13% (vol/vol) ethanol. One hundred kg blood-free placental tissue was recovered by pressing in order to thoroughly wash the tissue and to discard all the contaminating blood. Lipids were extracted by addition of 350 L *n*-butanol (13). The preparations were shaken for 4 h at −5°C and were further kept in *n*-butanol for 8 h at 2°C. The butanolic phase was separated by pressing, filtered and stored at −20°C in the presence of 3 g of BHT before concentration.

Purification of lipids. From an aliquot of the preparation, neutral lipids were first removed by preparative

thin-layer chromatography (TLC) using hexane/diethyl ether/acetic acid (60:40:1, by vol) as the developing solvent. Phospholipids were then eluted from the gel with chloroform/methanol/water (5:5:1, by vol), and dried by rotatory evaporation under vacuum at 30°C. Individual phospholipid classes were resolved by TLC on silica gel LK5 plates using chloroform/methanol/40% aqueous methylamine (60:20:5, by vol) as the developing solvent (14). Phospholipid fractions were eluted from the silica gel with chloroform/methanol/water (5:5:1, by vol) and were dried as described above. They were stored at −20°C under nitrogen in the presence of 5×10^{-5} M BHT, prior to fatty acid and molecular species analysis.

Quantitation of phospholipid classes by HPLC. Phospholipid classes were separated and quantified as described by Juanéda *et al.* (15). Briefly, the separation was achieved by normal-phase HPLC, using a 5 μm Lichrosorb Si60 silica column (250 × 7.5 mm) (Merck) and a four-solvent elution gradient (chloroform, hexane, 2-propanol, water). Quantitations were done with a Cunow light-scattering detector.

Separation of phospholipid subclasses. Choline glycerophospholipids (CPL), ethanolamine glycerophospholipids (EPL), serine glycerophospholipids (SPL) and inositol glycerophospholipids (IPL) were isolated on TLC plates as described above and converted to dinitrobenzoate derivatives following phospholipase C hydrolysis (16). Two hundred μg of phospholipids in 0.7 mL 30 mM H_3BO_3, 10 mM Tris-HCl buffer (pH 7.5) containing 0.02% BHT were sonicated for 5 min before addition of 50 U of phospholipase C overnight at 30°C under vigorous shaking. The resulting diradylglycerols were extracted with diethyl ether and converted into their dinitrobenzoyl derivatives by treatment with 3,5-dinitrobenzoyl chloride in anhydrous pyridine for 30 min at 60°C. After cooling, 1 mL H_2O was added to the samples and the products were extracted twice with hexane. The subclasses of dinitrobenzoyldiradylglycerols were further resolved by TLC on silica gel 60 G plates using toluene/hexane/diethyl ether (50:45:4, by vol) as solvent. The 1-alk-1′-enyl-2-acyl, 1-alkyl-2-acyl and 1,2-diacyl subclasses showed R_f values of 0.64, 0.58 and 0.38, respectively, and were separated. An equal amount (approximately 10 μg) of the 17:0/17:0 1,2-diacyl derivative was applied to each fraction as internal standard for the quantitation of molecular species peaks. The band of each subclass was scraped off and extracted twice with a mixture of diethyl ether/hexane (1:1, vol/vol). Because of internal standard coelution with 16:0/18:0 (1,2-diacyl) and 18:0/16:0 (1,2-diacyl), 1,2-diacyl subclasses were also prepared without adding 17:0/17:0 (1,2-diacyl) derivative.

Separation and quantitation of molecular species. Separation and quantitation of molecular species were accomplished by reverse-phase HPLC (16) using a 5 μm Ultrasphere ODS column (250 × 4.6 mm) (Beckman, Berkeley, CA) and an isocratic elution with acetonitrile/2-propanol (90:10, vol/vol) at a flow rate of 1.5 mL/min. The products were quantified by measuring absorbance at 240 nm. Molecular species were identified by comparison of their retention times with those of standards synthesized from known phospholipids and 1,2-diacylglycerols (17); the assignments were verified by the graphical representation method of Patton *et al.* (18).

Quantitation of CPL and EPL subclasses. The subclass compositions of CPL and EPL were calculated from total relative peak areas with respect to the internal standard used in each subclass. The data were confirmed by quantitative separation of the benzoyldiradylglycerol derivatives obtained from CPL and EPL by HPLC using a 5 μm Hypersil-H215 column (250 × 4.6 mm, Société Française Chromato Colonne, Neuilly-Plaisance, France) with cyclohexane/hexane/*t*-butyl methyl ether/acetic acid (375:125:12:0.1, by vol) as the mobile phase (19).

Gas-liquid chromatography (GLC) analysis. Fatty acid methyl esters were prepared by esterification with 14% boron trifluoride in methanol (20). The fatty acid composition of placental phospholipids was determined by GLC, using a Perkin-Elmer (Buckinghamshire, England) chromatograph model 5830, equipped with an SP 2380 capillary column (60 m × 0.25 mm) (Supelco, Bellefonte, PA) (17).

RESULTS

On average, 1.8 kg of total lipids were obtained by extraction of 100 kg of placentae tissue. The total phospholipid fraction accounted for more than 70 wt% of the extracted tissue lipids, whereas free fatty acids, triglycerides and cholesterol represented only 7, 3.5 and 19 wt%, respectively. Trace amounts of steryl esters were found in the butanolic lipid extract.

The composition of the phospholipid fraction of a batch of human placentae from European countries (Batch A) was analyzed in more detail. CPL, EPL and sphingomyelin were the major phospholipid classes, representing 53.19 ± 0.16, 14.60 ± 0.38 and 21.74 ± 0.40 wt%, respectively, of total phospholipids. IPL, SPL and cardiolipin accounted for only 4.39 ± 0.36, 2.88 ± 0.12 and 3.22 ± 0.15 wt% of total phospholipids. Phosphatidic acid, lysophosphatidylcholine and lysophosphatidylethanolamine were below the detection limit, accounting for less than 1 wt% of the total phospholipids. The composition of CPL and EPL was first analyzed by HPLC of each subclass by determining the absolute amount of molecular species relative to diheptadecanoylglycerol used as internal standard. CPL and EPL were also analyzed by straight-phase HPLC and their subclasses were quantitated by measuring UV absorption of each subclass at 240 nm. 1,2-Diacylglycerophosphocholine (GPC) was the major subclass of GPC and accounted for 91.69 ± 1.09 mole% of GPL, while 1-alkyl-2-acyl-GPC and 1-alk-1′-enyl-2-acyl-GPC represented 5.70 ± 0.64 and 2.61 ± 0.42 percent of this phospholipid class, respectively. The composition of EPL was quite different, with 1,2-diacyl and 1-alk-1′-enyl-2-acyl forms occurring at high proportions: 54.60 ± 1.15 mol% and 43.77 ± 1.15 mol% of the total class, respectively, whereas 1-alkyl-2-acyl-glycerophosphoethanolamine (GPE) was a minor component, with 1.63 ± 0.50 mol%.

In order to assess the adequate conservation of the placentae lipids, the fatty acid composition of total phospholipids was analyzed from five placental batches coming from different parts of the world (see Materials and Methods section). Within the five placentae batches, none of the main fatty acids differed by more than 10% from the mean of the five batches (Table 1). As no differences were observed between the fatty acids of the five batches by a X^2 test (contingency table analysis) (Statview, Macintosh), they were considered as having identical fatty acid

TABLE 1

Fatty Acid Analysis (mol%) of Total Phospholipids from Several Batches of Human Placenta[a]

	Batch					
	A (n = 3)	B (n = 3)	C (n = 3)	D (n = 3)	E (n = 3)	Mean (n = 15)
16:0 DMA	4.1 ± 0.0	4.4 ± 0.2	4.0 ± 0.2	4.3 ± 0.1	4.3 ± 0.0	4.2 ± 0.2
16:0	28.0 ± 0.0	29.7 ± 0.7	28.4 ± 0.2	28.2 ± 0.5	29.0 ± 0.4	28.6 ± 0.8
16:1n-9	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
16:1n-7	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
18:0 DMA	1.9 ± 0.0	2.4 ± 0.1	1.9 ± 0.0	2.0 ± 0.1	1.7 ± 0.0	2.0 ± 0.2
18:1n-9	9.0 ± 0.2	7.5 ± 0.7	8.0 ± 0.1	8.4 ± 0.1	7.9 ± 0.8	8.2 ± 0.7
18:1n-7	1.5 ± 0.0	1.4 ± 0.2	1.4 ± 0.0	1.5 ± 0.0	1.4 ± 0.2	1.4 ± 0.2
18:2n-6	8.7 ± 0.1	8.7 ± 0.0	8.6 ± 0.1	8.6 ± 0.0	8.6 ± 0.3	8.6 ± 0.2
18:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
20:2n-6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
22:0	0.9 ± 0.0	0.7 ± 0.2	0.8 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.7 ± 0.1
20:3n-6	4.1 ± 0.0	4.4 ± 0.1	3.9 ± 0.0	4.2 ± 0.0	4.3 ± 0.2	4.2 ± 0.2
20:4n-6	19.3 ± 0.0	19.5 ± 0.1	20.1 ± 0.1	18.2 ± 0.2	19.4 ± 0.2	19.3 ± 0.7
24:0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.8 ± 0.1
22:4n-6	1.5 ± 0.2	1.8 ± 0.0	1.8 ± 0.0	1.9 ± 0.0	1.7 ± 0.1	1.7 ± 0.2
24:1n-9	0.6 ± 0.2	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.2
22:5n-6	0.7 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.6 ± 0.0	1.5 ± 0.1	0.9 ± 0.3
22:5n-3	0.7 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
22:6n-3	3.5 ± 0.1	2.9 ± 0.2	3.5 ± 0.0	3.7 ± 0.0	2.8 ± 0.1	3.3 ± 0.4
SFA	49.1 ± 0.1	50.7 ± 1.3	49.3 ± 0.1	50.0 ± 0.4	50.4 ± 0.8	49.9 ± 0.9
MUFA	11.9 ± 0.0	10.1 ± 1.5	10.7 ± 0.0	11.0 ± 0.1	10.2 ± 1.3	10.8 ± 1.0
PUFA	39.0 ± 0.2	39.2 ± 0.2	39.9 ± 0.0	38.4 ± 0.2	39.1 ± 0.9	39.1 ± 0.7
n-6 PUFA	34.8 ± 0.3	35.8 ± 0.0	35.8 ± 0.1	34.0 ± 0.3	35.8 ± 0.7	35.3 ± 0.9
n-3 PUFA	4.2 ± 0.0	3.3 ± 0.2	4.1 ± 0.0	4.4 ± 0.0	3.3 ± 0.1	3.9 ± 0.5

[a]Results are expressed as means ± SD of three experiments. Abbreviations: DMA, dimethylacetal; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

composition, and phospholipid molecular species were analyzed on one batch, which was already a pool of 250 placentae. Fatty acid analyses performed on Batch A revealed that all phospholipid classes contain relatively high proportions of n-6 and n-3 long-chain PUFA, mainly 20:4n-6 and 22:6n-3, with 22:6n-3 accounting for most of the n-3 long-chain PUFA (Table 2). The greatest amount of long-chain PUFA was present in EPL (46.7 mol%) and in IPL (39.9 mol%). CPL and SPL were much richer in 18:1n-9 and 18:2n-6. The proportion of 22:6n-3 and 22:5n-3 was 5.9- and 8.7-fold higher in EPL than in CPL, respectively. The data in Tables 3 and 4 represent the first report on the glycerophospholipid molecular species of human placenta. The molecular species composition of the 1,2-diacyl glycerophospholipids were consistent with the fatty acid composition of each class (Table 3). In accordance with the fatty acid composition of SPL, 1,2-diacyl-glycerophosphoserines (GPS) contained a large amount of species with 18:1 at the *sn*-2 position, essentially associated with 18:0 (22.1 mol%) and 16:0 (5.3 mol%) at the *sn*-1 position. In contrast, in 1,2-diacyl-GPC and 1-alkyl-2-acyl-GPC, 18:1 was combined with 16:0 and 18:1 (12.9 and 19.0 mol%, respectively). 1,2-Diacyl-GPC, 1-alkyl-2-acyl-GPC and 1,2-diacyl-GPS were also characterized by high proportions of molecular species containing 18:2n-6. The resolution of 16:0/16:0 and 18:0/18:2n-6 was further achieved by GLC analysis of the HPLC fractions and showed that 16:0/16:0 was present only in trace amounts. High proportions of molecular species with arachidonic acid at the *sn*-2 position were found in all subclasses, especially in 1,2-diacyl-glycerophosphoinositols (GPI) and 1-alk-1′-enyl-2-acyl-GPE, where it was mainly associated with 18:0 (42.6 mol%) and 16:0 (24.0 mol%), respectively. Placenta diacyl-GPC contained high amounts of species with 20:3n-6 at the *sn*-2 position, 16:0/20:3n-6 and 18:0/20:3n-6 representing 17.3 and 12.9 mol% of 1,2-diacyl-GPS and 1,2-diacyl-GPI, respectively. 1-Alk-1′-enyl-2-acyl- and 1,2-diacyl-GPE were the subclasses containing the highest concentration of n-3 fatty acids, namely 22:6n-3 and 22:5n-3, although they were depleted in 18:3n-3 and 20:5n-3. Placentae EPL were rich in 22:6n-3, which occurs mainly as 16:0/22:6n-3 in plasmalogenic form and as 16:0/22:6n-3, 18:1/22:6n-3 and 18:0/22:6n-3 in 1,2-diacyl-GPE. 1-Alk-1′-enyl-2-acyl-GPE was also rich in 22 carbon fatty acids from the n-6 family, mainly 22:4n-6, which was present as 16:0/22:4n-6 (6.0 mol%) and 18:0/22:4n-6 (2.2 mol%).

DISCUSSION

Residual placenta lipids were found to be rich in n-6 and n-3 PUFA. They could be a potential source for the production of phospholipid molecular species enriched in these two fatty acid families. The lipid composition of placenta prepared from bulk tissue was found to be very close to that of individual placentae (21) whose lipids had been extracted by the classical Folch method. Proportions of lipid classes found in our preparations were comparable with those reported in the native tissue (21,22). On average, 1 kg of total phospholipids could be prepared from 100 kg of placentae. CPL, sphingomyelin and EPL occurred as the major phospholipid classes and their

TABLE 2

Fatty Acid Composition of Phospholipid Classes from Human Placenta Batch A[a]

	CPL	EPL	SPL	IPL
16:0 DMA	1.5 ± 0.0	11.4 ± 0.3	0.5 ± 0.1	n.d.
16:0	36.8 ± 0.2	6.5 ± 0.1	15.0 ± 1.3	17.8 ± 0.6
16:1n-9	0.6 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.3 ± 0.0
16:1n-7	0.7 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
18:0 DMA	0.2 ± 0.0	7.1 ± 0.2	n.d.	n.d.
18:1n-9 DMA	n.d.	1.2 ± 0.1	n.d.	n.d.
18:1n-7 DMA	n.d.	0.4 ± 0.0	n.d.	n.d.
18:0	8.9 ± 0.1	14.4 ± 0.3	37.4 ± 0.5	31.4 ± 0.3
18:1n-9	9.5 ± 0.0	7.4 ± 0.1	15.2 ± 0.1	4.9 ± 0.1
18:1n-7	2.3 ± 0.0	0.5 ± 0.0	1.1 ± 0.0	1.1 ± 0.1
18:2n-6	14.2 ± 0.1	3.6 ± 0.2	5.4 ± 0.2	2.5 ± 0.0
18:3n-6	n.d.	0.1 ± 0.0	n.d.	n.d.
20:1n-9	n.d.	n.d.	n.d.	0.5 ± 0.1
20:2n-6	0.4 ± 0.0	0.1 ± 0.0	n.d.	n.d.
22:0	n.d.	n.d.	0.6 ± 0.1	0.7 ± 0.1
20:3n-6	4.6 ± 0.0	3.4 ± 0.1	7.5 ± 0.3	7.1 ± 0.1
20:4n-6	18.0 ± 0.3	28.0 ± 0.3	10.4 ± 0.4	29.2 ± 0.4
20:5n-3	n.d.	0.1 ± 0.0	n.d.	n.d.
24:0	n.d.	n.d.	0.4 ± 0.0	1.0 ± 0.1
22:4n-6	0.5 ± 0.0	3.2 ± 0.1	2.1 ± 0.3	0.6 ± 0.0
24:1n-9	n.d.	n.d.	n.d.	0.3 ± 0.1
22:5n-6	0.2 ± 0.0	1.8 ± 0.0	0.9 ± 0.0	0.4 ± 0.1
22:5n-3	0.2 ± 0.0	1.7 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
22:6n-3	1.6 ± 0.0	8.5 ± 0.0	2.6 ± 0.1	1.4 ± 0.0
SFA	47.3 ± 0.3	39.3 ± 1.0	53.8 ± 2.0	51.0 ± 1.0
MUFA	13.0 ± 0.2	9.7 ± 0.3	16.9 ± 0.2	7.4 ± 0.2
PUFA	39.7 ± 0.5	50.5 ± 0.7	29.3 ± 1.4	41.6 ± 0.6
n-6 PUFA	38.0 ± 0.5	40.2 ± 0.7	26.3 ± 1.2	39.8 ± 0.6
n-3 PUFA	1.8 ± 0.0	10.3 ± 0.1	3.1 ± 0.2	1.9 ± 0.1

[a]Results are expressed as means ± SD of three experiments. Abbreviations: CPL, choline glycerophospholipids; EPL, ethanolamine glycerophospholipids; IPL, inositol glycerophospholipids; SPL, serine glycerophospholipids. Other abbreviations, see Table 1; n.d., not detected.

TABLE 3

Molecular Species Composition (mol%) of 1,2-Diacyl-GPC, GPE, GPS and GPI from the Human Placenta Batch A[a]

Molecular species	1,2-Diacyl-GPC	1,2-Diacyl-GPE	1,2-Diacyl-GPS	1,2-Diacyl-GPI
16:1/22:6n-3 18:2/22:6n-3	0.48 ± 0.04	0.27 ± 0.02	n.d.	n.d.
16:1/20:4n-6 18:2/20:4n-6	2.05 ± 0.12	1.18 ± 0.05	n.d.	0.71 ± 0.03
18:1/20:5n-3 16:0/20:5n-3	n.d.	2.00 ± 0.08	n.d.	n.d.
18:1/22:6n-3 16:0/22:6n-3	5.15 ± 0.07 1.30 ± 0.09	4.05 ± 0.15 3.51 ± 0.02	1.44 ± 0.06	1.96 ± 0.10
18:1/20:4n-6 16:0/22:5n-3	2.86 ± 0.22	5.76 ± 0.02	0.81 ± 0.05	4.12 ± 0.13
16:0/20:4n-6	17.90 ± 0.95	11.78 ± 0.24	3.71 ± 0.03	19.41 ± 0.10
18:1/22:5n-6	n.d.	0.55 ± 0.05	n.d.	n.d.
16:0/22:5n-6	0.13 ± 0.02	0.72 ± 0.10	0.25 ± 0.02	0.33 ± 0.03
18:1/18:2n-6	3.75 ± 0.14	2.42 ± 0.08	0.66 ± 0.01	0.73 ± 0.02
16:0/18:2n-6 18:0/22:6n-3 18:1/20:3n-6	16.88 ± 0.09	10.73 ± 0.25	8.43 ± 0.10	4.14 ± 0.15
16:0/20:3n-6	6.00 ± 0.06	2.93 ± 0.13	2.82 ± 0.08	4.85 ± 0.07
16:0/22:4n-6	0.42 ± 0.04	0.93 ± 0.02	0.78 ± 0.08	0.77 ± 0.08
18:0/22:5n-3	n.d.	0.60 ± 0.08	0.96 ± 0.04	0.66 ± 0.04
18:0/20:4n-6	7.55 ± 0.16	27.92 ± 0.68	16.58 ± 0.28	42.57 ± 0.59
18:0/22:5n-6	n.d.	1.43 ± 0.03	2.18 ± 0.04	0.76 ± 0.09
18:1/18:1 16:0/18:1	12.92 ± 0.37	1.71 ± 0.10 2.70 ± 0.14	0.87 ± 0.03 5.32 ± 0.18	0.34 ± 0.05 3.43 ± 0.15
18:0/18:2n-6 16:0/16:0	17.15 ± 0.35	6.23 ± 0.10	11.27 ± 0.18	3.16 ± 0.07
18:0/20:3n-6	2.09 ± 0.11	7.18 ± 0.41	14.52 ± 0.09	8.08 ± 0.17
18:0/22:4n-6	0.37 ± 0.05	2.19 ± 0.10	4.18 ± 0.12	1.59 ± 0.16
18:0/18:1	1.98 ± 0.05	3.24 ± 0.14	22.07 ± 0.04	2.44 ± 0.14
16:0/18:0 18:0/16:0	1.06 ± 0.10	n.d.	2.12 ± 0.17	n.d.
18:0/18:0	n.d.	n.d.	1.04 ± 0.04	n.d.

[a]Results are expressed as means ± SD of three experiments. Abbreviations: GPC, glycerophosphocholines; GPE, glycerophosphoethanolamines; GPS, glycerophosphoserines; GPI, glycerophosphoinositols; n.d., not detected.

proportions were very close to the values reported for native tissues (21–24). However, the amount of SPL (2.9%) was lower than that reported by Chirouze *et al.* (21) (4.4%) and Nelson (23) (8.7%). This could indicate some selectivity in the large scale butanolic extraction of phospholipid classes. The subclass compositions of CPL and EPL were similar to those of several human tissues, including vascular cells (25), with high proportions of the 1-alk-1′-enyl-2-acyl form in EPL, and the 1,2-diacyl form accounting for more than 90% of CPL. The origin of placenta had no influence on the proportion of PUFAs which was constant within the five placenta phospholipid preparations analyzed and was close to that reported by Chirouze *et al.* (21) and Percy *et al.* (24), suggesting negligible fatty acid degradation during placenta storage and large scale processing of the lipid preparation. The molecular species distribution of human placenta phospholipids resembles that reported for human vascular cells (17,26). 1,2-Diacyl-GPI and 1-alk-1′-enyl-2-acyl-GPE were the subclasses containing the highest proportions of 18:0/20:4n-6. The molecular species with n-6 and n-3 long-chain PUFAs were mostly associated with 1-alk-1′-enyl-2-acyl-GPE. 1,2-Diacyl-GPC contained higher amounts of 18:2n-6 and 18:1. Taking into account that 1,2-diacyl-GPC represents 45 wt% of the total phospholipids, 450 g of molecular species with a fatty acid of nutritional interest at the *sn*-2 position could be purified from 100 kg of placenta. 1,2-Diacyl-GPE and 1-alk-1′-enyl-2-acyl-GPE, which are rich in 20:4n-6 and 22:6n-3, could also be prepared by large-scale processing.

Adequate extraction of placenta CPL, EPL and IPL was achieved by the large scale isolation process. These phospholipids are mostly enriched in PUFAs. The large scale purification of phospholipid classes would allow for production of fractions enriched in n-6 fatty acids, mainly 20:3n-6, 20:4n-6 and 22:4n-6, and fractions enriched in n-3 fatty acids, such as 22:5n-3 and 22:6n-3.

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TABLE 4

Molecular Species Composition (mol%) of 1-Alkyl-2-acyl-GPC and 1-Alk-1′-enyl-2-acyl-GPE from Human Placenta Batch A[a]

Molecular species	1-Alkyl-2-acyl-GPC	1-Alk-1′-enyl-2-acyl-GPE
16:1/20:4n-6 18:2/20:4n-6	0.90 ± 0.13	n.d.
18:1/20:5n-3 16:0/20:5n-3	n.d.	0.42 ± 0.04
18:1/22:6n-3	1.61 ± 0.01	1.26 ± 0.05
16:0/22:6n-3	2.79 ± 0.16	11.70 ± 0.19
18:1/20:4n-6 16:0/22:5n-3	10.05 ± 0.14	8.65 ± 0.33
16:0/20:4n-6	14.47 ± 1.30	24.04 ± 0.55
16:0/22:5n-6	0.77 ± 0.05	1.94 ± 0.09
18:1/18:2n-6	2.95 ± 0.14	1.46 ± 0.10
16:0/18:2n-6 18:0/22:6n-3 18:1/20:3n-6	6.51 ± 0.10	7.98 ± 0.08
16:0/20:3n-6	1.54 ± 0.03	1.22 ± 0.04
16:0/22:4n-6	1.42 ± 0.08	5.98 ± 0.10
18:0/22:5n-3	n.d.	1.37 ± 0.02
18:0/20:4n-6	4.92 ± 0.02	22.01 ± 0.20
18:0/22:5n-6	n.d.	0.94 ± 0.09
18:1/18:1 16:0/18:1	19.02 ± 0.35	0.97 ± 0.06 4.38 ± 0.05
18:0/18:2n-6 16:0/16:0	25.98 ± 1.01	1.11 ± 0.07
18:0/20:3n-6	0.76 ± 0.04	0.98 ± 0.05
18:0/22:4n-6	1.41 ± 0.06	2.22 ± 0.10
18:0/18:1	1.04 ± 0.06	1.36 ± 0.06
16:0/18:0 18:0/16:0	3.46 ± 0.05	1.46 ± 0.07

[a]Results are expressed as means ± SD of three experiments. See Table 3 for abbreviations.

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Triacylglycerol Structure of Human Colostrum and Mature Milk

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Because triacylglycerol (TAG) structure influences the metabolic fate of its component fatty acids, we have examined human colostrum and mature milk TAG with particular attention to the location of the very long chain polyunsaturated fatty acid on the glycerol backbone. The analysis was based on the formation of various diacylglycerol species from human milk TAG upon chemical (Grignard degradation) or enzymatic degradation. The structure of the TAG was subsequently deduced from data obtained by gas chromatographic analysis of the fatty acid methyl esters in the diacylglycerol subfractions. The highly specific TAG structure observed was identical in mature milk and colostrum. The three major fatty acids (oleic, palmitic and linoleic acids) each showed a specific preference for a particular position within milk TAG: oleic acid for the *sn*-1 position, palmitic acid for the *sn*-2 position and linoleic acid for the *sn*-3 position. Linoleic and α-linolenic acids exhibited the same pattern of distribution and they were both found primarily in the *sn*-3 (50%) and *sn*-1 (30%) positions. Their longer chain analogs, arachidonic and docosahexaenoic acids, were located in the *sn*-2 and *sn*-3 positions. These results show that polyunsaturated fatty acids are distributed within the TAG molecule of human milk in a highly specific fashion, and that in the first month of lactation the maturation of the mammary gland does not affect the milk TAG structure. ***Lipids 28,*** **637–643 (1993).**

Breast milk is considered the optimal form of nutrition for infants (1) and contains both parent essential fatty acids (linoleic acid and α-linolenic acid) and very long chain polyunsaturated fatty acids (VLC-PUFA, *i.e.*, PUFA ⩾ 20 carbons). A balanced amount of these fatty acids is required for normal maturation and function of the nervous system (2–5), as well as for adequate production of eicosanoids (2,3). Several expert committees now recommend the addition of both VLC-PUFA and α-linolenate to infant formulas (6,7).

The structure of the triacylglycerols (TAG) used in commercially prepared formulas influences the pre- and post-enterocyte bioavailability of their constituent fatty acids. For example, the presence of some VLC-PUFAs, such as 20:4n-6, 20:5n-3 and 22:6n-3, at the outer positions of TAG induces resistance to pancreatic lipase hydrolysis *in vitro* (8,9). In addition, the distribution of fatty acid between the outer and the *sn*-2 position in the glycerol molecule governs the lumenal partition between the free and the 2-monoacyl-*sn*-glycerol form (10–12), and subsequently modifies the rate of intestinal fatty acid uptake. The absorption of palmitic acid is improved when it is located in the *sn*-2 position of TAG as compared to the *sn*-1,3 positions (13,14). A similar difference in absorption was observed for stearic acid (15). The molecular form of fatty acid taken up by the enterocyte also modulates its incorporation into either the TAG or the phospholipids fraction of chylomicrons (16), as well as its positional distribution in the TAG of chylomicrons (16–18). As the fatty acids are differently metabolized whether bound primarily to the TAG or phospholipid fraction of chylomicrons (19,20), and because the post-heparin plasma lipoprotein lipase preferentially hydrolyzes the ester bond in the *sn*-1 position (21), the post enterocyte metabolic fate of component fatty acids is expected to depend on the structure of the ingested TAG. For instance, the positional structure of TAG species affects both the fractional plasma clearance rates of chylomicrons and the recoveries of TAG in the liver and spleen (22), modulates the atherogenic features of a given oil (23) and may effect the pharmacological actions of dietary n-3 VLC-PUFA used for therapeutic purposes (24,25). Because breast milk is the natural source of fat for the newborn, the structure of its TAG may be used as a biological reference point. However, the proportion of VLC-PUFA in each position of the glycerol backbone has not been carefully determined in human milk TAG. Furthermore, whether the TAG structure changes with maturation of the mammary gland along with changes observed in the fatty acid composition (26–28) is unknown. To address these questions, we have examined the distribution of fatty acids in human colostrum and mature milk TAG.

MATERIALS AND METHODS

Subjects and milk collection. Fifteen Caucasian, healthy mothers living in the Loire Valley area of France volunteered for this study. Women taking drugs, including alcohol, or with self-selected diets (vegetarian, vegan, food fad) or with complications of pregnancy, including cesarian section, were not included in the study. All mothers were on their usual diets and no dietary modifications were proposed. Milk samples (4–5 mL) were taken prior to the mother's lunch. Colostrum was obtained on day 5 and mature milk on day 30 after delivery. All samples were immediately frozen and then stored at −70°C within one hour. The protocol was approved by the Ethics Committee of our institution. Lipid analyses were performed within a week following milk collection.

Chemicals and reagents. All solvents (chloroform, hexane, heptane, diethyl ether and di-isopropyl ether) were of high-performance liquid chromatography grade. Solvents and glacial acetic acid were purchased from Carlo Erba (Milan, Italy). Diethyl ether was purchased in small amounts and used within weeks to minimize peroxidation. Methanol and ammonia (25%) were from Prolabo (Vitry sur Seine, France). Pyridine and toluene were from Merck (Darmstadt, Germany). Fatty acid methyl ester standards were from Nu-Chek-Prep (Elysian, MN) or Supelco

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Abbreviations: BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; GPC, glycerophosphocholine; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol; TLC, thin-layer chromatography; VLC-PUFA, very long chain polyunsaturated fatty acids.

(Bellefonte, PA). Tri-tridecanoin, 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine (GPC), 2,3-di-hexadecanoyl-*sn*-glycero-1-phosphocholine, choline chloride, butylated hydroxytoluene (BHT) and boron trifluoride in methanol were purchased from Sigma (St. Louis, MO). Boric acid and phosphorus oxychloride were purchased from Merck and ethyl magnesium bromide from Aldrich (Milwaukee, WI). Thin-layer chromatography (TLC) plates (type LK6 plates, 250 μm layer, 5 × 20 cm) were from Whatman (Maidstone, England). Porcine pancreatic lipase (EC 33.1.1.3, type VI-S) and phospholipase C from *C. welchii* (EC 3.1.4.3, type I) were from Sigma. All chemical and enzymatic reactions were carried out under nitrogen, with BHT added as an antioxidant (0.005%, wt/vol). BHT was also added to all TLC developing solvents (0.05%, wt/vol), except as noted.

TAG quantification. In order to carry out each stereospecific analysis with an equal amount of TAG and to compensate for variations in milk fat content between the individuals, the TAG concentration in each milk sample was measured. Tri-tridecanoin (150 μg) was added to 3 mL of milk as an internal standard. Lipids were then extracted as previously described (29) and a measured aliquot was used to purify TAG by TLC on silica gel plates. Hexane/diethyl ether/acetic acid (75:25:1, by vol) without BHT was used as the developing solvent. TLC plates were sprayed with 2′,7′-dichlorofluorescein (2% in ethanol), and lipid fractions were visualized under ultraviolet light and identified by comparison of their R_f values with those of authentic standards. Quantification of TAG was achieved by gas chromatography after conversion of TAG to fatty acid methyl esters. The internal tridecanoic acid methyl ester was used as the standard for both fatty acid and TAG quantification, and the mass of fatty acid methyl esters was considered as a close approximation of that of TAG. The milk volume per sample was then adjusted to arrive at the desired amount of TAG in each sample.

Fatty acid analysis. Fatty acids were converted to the methyl esters with 14% (wt/vol) boron trifluoride in methanol (30). All reactions were carried out under nitrogen. Fatty acid methyl esters were analyzed by gas chromatography (Carlo Erba type GC 6000, Vega series 2) using a 50 m × 0.32 mm i.d. fused silica capillary column with a 0.25 μm film of carbowax 20 M (Star, Cergy-Pontoise, France), an on-column injector and a flame-ionization detector connected to a digital integrator (Spectraphysics SP4270, San Jose, CA). Operating conditions have been detailed previously (29). The areas of nonidentified peaks were less than 2%. Results were expressed in mol% of the identified fatty acid methyl esters after conversion of the respective peak areas. Inter- and intra-assay coefficients of variation have been evaluated previously (29).

Stereospecific analysis of milk TAG. This method is based upon the random generation of 1,3-diacyl-*sn*-glycerol along with an equimolar mixture of 1,2- and 2,3-diacyl-*sn*-glycerols (*rac*-1,2-diacylglycerols) by Grignard degradation of TAG. *rac*-1,2-Diacylglycerols were converted to *rac*-phosphatidylcholines by chemical means, and the 1,2-diacyl-*sn*-glycero-3-phosphocholine was selectively hydrolyzed by phospholipase C. This allowed the separation of enantiomeric 1,2-diacyl-*sn*-glycerol from the remaining 2,3-diacyl-*sn*-glycero-1-phosphocholine as described by Myher and Kuksis (31).

In brief, 30 mg of TAG were purified from the milk total lipid extracts by preparative TLC on boric acid-impregnated plates (by submerging the TLC plates in a 5% methanolic solution for 5 min) in order to minimize isomerization. The TAG fraction was eluted from the gel with chloroform/methanol/water (4:4:3.8, by vol). The TAGs were dried by evaporation after ethanol addition, dissolved in 1.2 mL of anhydrous diethyl ether, transferred to a glass flask and stirred under nitrogen at room temperature. Ethyl magnesium bromide (3 M in diethyl ether, 0.1 mL) was added to the TAG solution, and the reaction was stopped after 25 s by addition of acetic acid/diethyl ether (1:9, vol/vol). The mixture was thoroughly washed as described (31), and dried. The *rac*-1,2-diacylglycerols were separated from the 1,3-diacyl-*sn*-glycerols on borate-impregnated silica gel plates using chloroform/acetone (97:3, vol/vol) as developing solvent (31). The fatty acids from the 1,3-diacyl-*sn*-glycerol and from an aliquot of the *rac*-1,2-diacylglycerols were converted to fatty acid methyl esters (30) for gas chromatographic analysis.

For the synthesis of *rac*-phosphatidylcholines, the remaining *rac*-1,2-diacylglycerols were evaporated to dryness from ethanol and dissolved in 0.65 mL of a mixture of chloroform/pyridine/phosphorus oxychloride at 0°C (47.5:47.5:5, by vol). The mixture was allowed to stand at 0°C for 1 h and then at 25°C for 1 h (31). Choline chloride (200 mg, acetone-dried powder) was added, and the mixture was stirred at 30°C for 15 h. After addition of 20 μL of water and 30 min of subsequent stirring, the volume was reduced under nitrogen, and the reaction products were extracted and thoroughly dried (31). The *rac*-phosphatidylcholines were separated from the unreacted *rac*-1,2-diacylglycerols by TLC on borate-impregnated silica gel plates (31). The fatty acid composition of the *rac*-1,2-diacylglycerols and of the *rac*-phosphatidylcholines were identical, ensuring that the synthetic process did not alter the fatty acid composition nor did selective reaction occur with respect to particular diacylglycerol species.

1,2-Diacyl-*sn*-glycero-3-phosphocholines were stereospecifically hydrolyzed from the *rac*-phosphatidylcholine fraction with phospholipase C, as previously described (31). The resulting 1,2-diacyl-*sn*-glycerols and 2,3-diacyl-*sn*-glycero-1-phosphocholines were resolved by TLC on borate-impregnated K6 plates using chloroform/methanol/acetic acid (20:90:10, by vol) as the developing solvent. Specificity of phospholipase C used for the *sn*-3 position of the phosphocholine group was ascertained using 1,2-pentadecanoyl-*sn*-glycero-3-phosphocholine and 2,3-hexadecanoyl-*sn*-glycero-1-phosphocholine as substrates.

Hydrolysis of milk TAG with pancreatic lipase. 2-Monoacyl-*sn*-glycerols were obtained from milk TAG upon hydrolysis of the acyl group in the 1- and 3- positions of glycerol using porcine pancreatic lipase (31). Purified milk TAGs (2.5 mg) were resuspended by vigorous shaking in 0.5 mL of TRIS/HCl buffer (1 M, pH 8.0) containing gum Arabic (10%) and 14 μL of a $CaCl_2$ solution (45% in water). The solution was submitted to pancreatic lipase hydrolysis (5000 units dissolved in 0.5 mL buffer, 10 min at 37°C with vigorous stirring). The reaction products were extracted with diethyl ether and the 2-monoacyl-*sn*-glycerols purified on K6 borate-impregnated plates with heptane/iso-propyl ether/acetic acid (60:40:4, by vol) as developing solvent. Fatty acid composition was

determined by gas chromatography of the fatty acid methyl esters (29).

Determination of the TAG structure. The structure of the original TAG was calculated from the fatty acid composition of each lipid fraction (1,2-diacyl-*sn*-glycerol, 2,3-diacyl-*sn*-glycero-1-phosphocholine and 1,3-diacyl-*sn*-glycerol). The proportion of individual fatty acids found in the three positions of the TAG was calculated according to the following equations (32):

$$2\text{-}sn\ (\text{mol}\%) = 2 \times rac\text{-}1,2\text{-diacylglycerol}\ (\text{mol}\%) - 1,3\text{-diacyl-}sn\text{-glycerol}\ (\text{mol}\%) \quad [1]$$

$$1\text{-}sn\ (\text{mol}\%) = 2 \times 1,2\text{-diacyl-}sn\text{-glycerol}\ (\text{mol}\%) - 2\text{-}sn\ (\text{mol}\%) \quad [2]$$

$$3\text{-}sn\ (\text{mol}\%) = 2 \times 2,3\text{-diacyl-}sn\ \text{GPC}\ (\text{mol}\%) - 2\text{-}sn\ (\text{mol}\%) \quad [3]$$

The 2-position fatty acid composition was also directly obtained by analysis of the 2-monoacyl-*sn*-glycerol fraction produced after pancreatic lipase hydrolysis of TAG.

Statistical analysis. Data were computed using Statview 512+® (Brain Power Inc., Calabassas, CA). Results are presented as the means ± SD (mol%). The content of each fatty acid found in the *sn*-2 position by the pancreatic lipase method or deduced from the diacylglycerol species produced by Grignard degradation was compared by the Mann and Whitney signed rank test. The P value was set at 0.05.

RESULTS

Under our experimental conditions, the theoretical and observed ratios of selected fatty acids in *rac*-1,2- and in 1,3-diacyl-*sn*-glycerols obtained after Grignard degradation of TAG standards were very similar (Table 1).

For all milk fatty acids except 18:0, the relative proportion found in the *sn*-2 position was not statistically different whether determined by pancreatic lipase hydrolysis or calculated from the diacylglycerol species produced by Grignard degradation. However the difference was close to significant for 22:6n-3 (1.07% and 0.83%, with the pancreatic lipase method and the Grignard degradation method, respectively; n = 13; $P = 0.06$). The gas chromatographic quantification of lipid fractions obtained after phospholipase C action on standards showed that 98.9% of the 1,2-diacyl-*sn*-glycero-3-phosphocholines were hydrolyzed to produce 1,2-diacyl-*sn*-glycerol, whereas only 1.13% of the 2,3-diacyl-*sn*-glycero-1-phosphocholines were hydrolyzed to 2,3-diacyl-*sn*-glycerol and phosphocholine.

The positional distribution of fatty acids in TAG from human colostrum is presented in Table 2. The *sn*-1 position was esterified predominantly with 18:1 (48%), 18:2n-6 (14%), 16:0 (13%) and 18:0 (11%) fatty acids. The *sn*-2 position was esterified primarily with 16:0 (54%), 18:1 (13%), 14:0 (11%) and 18:2n-6 (8%) fatty acids. The *sn*-3 position was principally composed of 18:1 (37%), 18:2n-6 (17%), 16:0 (11%) and 14:0 (10%) fatty acids.

The relative distribution of each saturated fatty acid (from 10:0 to 18:0) at the three sites of esterification is shown in Figure 1. The proportion of 10:0 to 16:0 fatty acids found in the *sn*-2 position increased with the length of the carbon chain with a commensurate decrease in the *sn*-3 position. Conversely, 64% of stearic acid was found in the *sn*-1 position, 26% in the *sn*-3 position and only 9% in the *sn*-2 position. The profile of the positional distribution of major monounsaturated fatty acids is depicted in Figure 2. The profile observed for 18:1 fatty acids was similar to the one seen for its saturated homologue (see Figs. 1 and 2). The 18-carbon essential fatty acids (18:2n-6

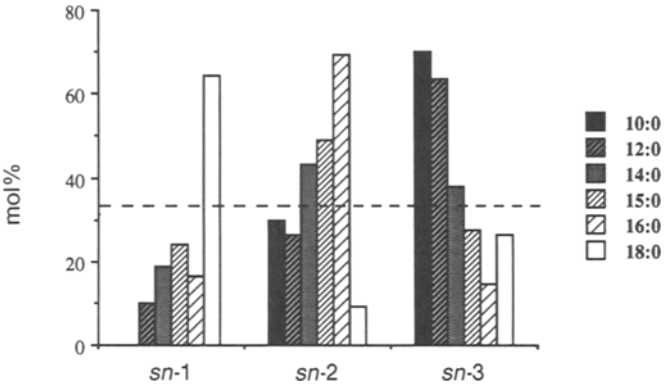

FIG. 1. Positional distribution of short-chain (C_{10}), medium-chain (C_{12} and C_{14}) and major long-chain ($\geq C_{16}$) saturated fatty acids in colostrum triacylglycerols. The horizontal dashed line indicates the proportion of each fatty acid expected on the basis of a random positional distribution (33.3%).

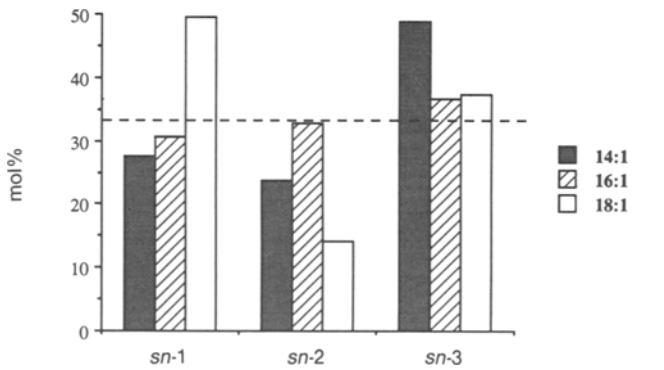

FIG. 2. Positional distribution of major monounsaturated fatty acids in colostrum triacylglycerols. The horizontal dashed line indicates the proportion of each fatty acid expected on the basis of a random positional distribution (33.3%).

TABLE 1

Theoretical and Experimental Ratios of Selected Fatty Acids in *rac*-Diacylglycerols and 1,3-Diacyl-*sn*-glycerols After Grignard Degradation of Triacylglycerol Standards

	rac-1,2-Diacylglycerols			1,3-Diacyl-*sn*-glycerols		
Triacylglycerols[a]	16:0/18:1		18:1/16:0	16:0/18:1		18:1/16:0
18:1/16:0/18:1	1[b]	0.96[c]	—	0	0.003	—
16:0/18:1/16:0	—		1 1.03	—		0 0.033
18:1/18:1/16:0	0.33	0.30	—	1	0.93	—

[a]Racemic mixture of isomers.
[b]Theoretical values are listed in the first column for each ratio analyzed.
[c]Experimental values are listed in the second column for each ratio analyzed.

TABLE 2

Results (mol%; means ± SD) of the Stereospecific Analysis of Colostrum Triacylglycerols (TAG) (n = 11)

Fatty acids	*sn*-1 (%)	*sn*-2 (%)	*sn*-3 (%)	Calculated TAG (%)[a]	Original TAG (%)
Saturated					
10:0	0.00	0.36 ± 0.34	0.84 ± 0.41	0.40 ± 0.15	0.56 ± 0.13
12:0	1.41 ± 1.1	3.81 ± 1.32	9.11 ± 3.22	4.78 ± 1.41	4.54 ± 1.05
14:0	4.80 ± 1.90	11.08 ± 2.20	9.73 ± 2.91	8.54 ± 2.11	7.41 ± 1.63
15:0	0.34 ± 0.22	0.69 ± 0.22	0.39 ± 0.12	0.47 ± 0.10	0.40 ± 0.08
16:0	12.58 ± 3.38	53.50 ± 3.21	11.21 ± 3.33	25.76 ± 2.33	26.22 ± 1.70
17:0	0.49 ± 0.13	0.33 ± 0.04	0.31 ± 0.13	0.38 ± 0.09	0.37 ± 0.05
18:0	11.35 ± 1.34	1.65 ± 0.42	4.67 ± 1.08	5.89 ± 0.67	6.65 ± 0.63
20:0	0.14 ± 0.08	0.15 ± 0.05	0.17 ± 0.08	0.15 ± 0.04	0.17 ± 0.04
Monounsaturated					
14:1n-5	0.22 ± 0.22	0.19 ± 0.13	0.39 ± 0.14	0.27 ± 0.08	0.18 ± 0.06
16:1[b,d]	2.93 ± 1.05	3.15 ± 0.64	3.52 ± 0.83	3.20 ± 0.62	2.80 ± 0.51
18:1[b,d]	48.43 ± 5.54	13.84 ± 1.58	36.62 ± 3.35	32.62 ± 2.20	34.40 ± 3.07
20:1[c]	0.74 ± 0.37	0.42 ± 0.21	0.72 ± 0.32	0.63 ± 0.25	0.70 ± 0.20
22:1n-9	0.44 ± 0.38	0.12 ± 0.05	0.45 ± 0.33	0.34 ± 0.23	0.14 ± 0.04
24:1n-9	0.00	0.00	0.11 ± 0.15	0.00	0.07 ± 0.05
n-6 Polyunsaturated					
18:2n-6[d]	14.05 ± 3.65	8.42 ± 2.22	17.25 ± 3.89	13.24 ± 3.18	13.13 ± 3.11
20:2n-6	0.60 ± 0.19	0.28 ± 0.11	0.73 ± 0.22	0.54 ± 0.14	0.67 ± 0.13
22:2n-6	0.00	0.12 ± 0.05	0.00	0.00	0.10 ± 0.08
20:3n-6	0.37 ± 0.17	0.25 ± 0.08	0.50 ± 0.24	0.37 ± 0.14	0.39 ± 0.12
20:4n-6	0.17 ± 0.16	0.74 ± 0.16	0.74 ± 0.22	0.55 ± 0.17	0.59 ± 0.11
22:4n-6	0.00	0.34 ± 0.08	0.15 ± 0.10	0.16 ± 0.12	0.19 ± 0.07
n-3 Polyunsaturated					
18:3n-3	0.66 ± 0.15	0.41 ± 0.10	1.03 ± 0.21	0.70 ± 0.25	0.68 ± 0.13
20:3n-3	0.05 ± 0.09	0.00	0.05 ± 0.09	0.00	0.00
18:4n-3	0.12 ± 0.30	0.06 ± 0.09	0.20 ± 0.29	0.12 ± 0.21	0.23 ± 0.11
20:4n-3	0.00	0.00	0.05 ± 0.09	0.00	0.00
20:5n-3	0.00	0.06 ± 0.07	0.11 ± 0.20	0.06 ± 0.10	0.06 ± 0.12
22:5n-3	0.00	0.34 ± 0.18	0.15 ± 0.14	0.16 ± 0.10	0.19 ± 0.11
22:6n-3	0.10 ± 0.12	0.74 ± 0.65	0.62 ± 0.55	0.49 ± 0.40	0.52 ± 0.35

[a](*sn*-1 + *sn*-2 + *sn*-3)/3 was used for the calculated triacylglycerols.
[b]n-7 + n-9 Isomers.
[c]n-9 + n-11 Isomers.
[d]Includes *trans* isomers.

and 18:3n-3) had identical distribution profiles at the glycerol molecule (Fig. 3). Their longer chain analogues, 20:4n-6 and 22:6n-3 (Fig. 4A) and 22:4n-6 and 22:5n-3 (Fig. 4B), were similar.

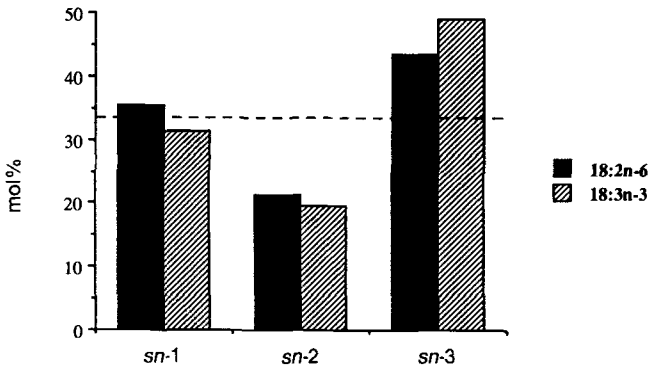

FIG. 3. Positional distribution of 18-carbon essential fatty acids in colostrum triacylglycerols. The horizontal dashed line indicates the proportion of each fatty acid expected on the basis of a random positional distribution (33.3%).

Although the VLC-PUFA content decreased from colostrum to mature milk, a similar positional distribution of each individual fatty acid in TAG was observed (see Tables 2 and 3).

DISCUSSION

This study demonstrates that in human milk TAG, the VLC-PUFAs, especially 20:4n-6 and 22:6n-3, are found primarily esterified in the *sn*-2 and *sn*-3 positions and that, more generally, the highly specific positional distribution of fatty acids is maintained throughout the first month of lactation.

Several authors (31,33,34) found that Grignard degradation caused significant acyl migration in the diacylglycerol moiety, especially for the 1,3-diacyl-*sn*-glycerols. Under our experimental conditions, the good agreement between theoretical and experimental 18:1/16:0 or 16:0/18:1 ratios found in the 1,2(2,3)-diacyl-*sn*-glycerols and in the 1,3-diacyl-*sn*-glycerols generated from TAG standards (Table 1) showed that Grignard degradation did not lead to pronounced acyl migration. For all the milk fatty acids but stearate, the content found in the *sn*-2 position was similar whether calculated from diacylglycerols produced

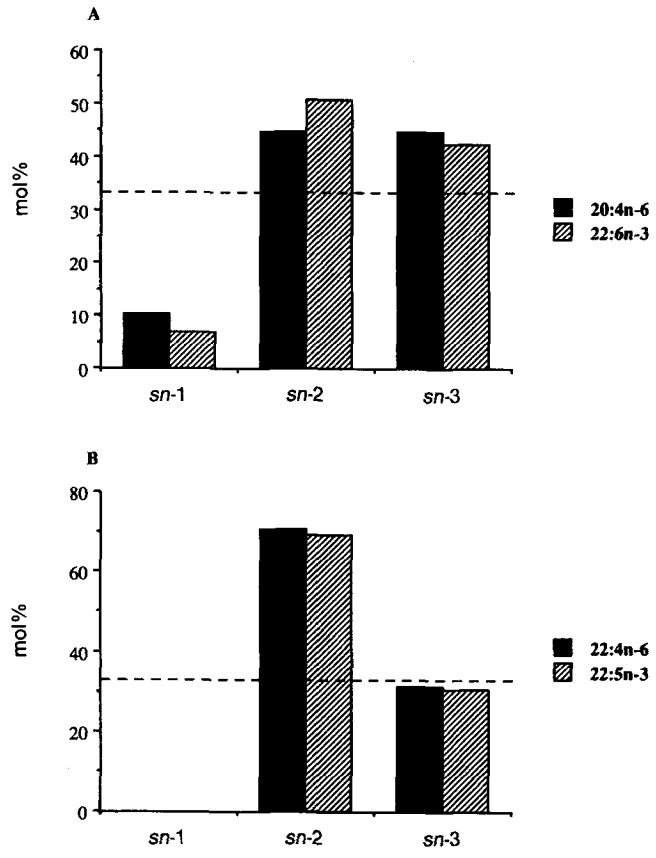

FIG. 4. Positional distribution of selected 20 and 22-carbon polyunsaturated fatty acids in colostrum triacylglycerols. The horizontal dashed line indicates the proportion of each fatty acid expected on the basis of a random positional distribution (33.3%).

by Grignard degradation (*i.e.*, 1,2(2,3)-diacyl-*sn*-glycerols and 1,3-diacyl-*sn*-glycerols) or determined after pancreatic lipase action on TAG. The difference found for stearic acid between the two methods has been previously described by Lawson and Hughes (35). It is known that some VLC-PUFA (20:4n-6, 20:5n-3 and 22:6n-3) are resistant to pancreatic lipase hydrolysis when linked to the outer positions of glycerol. Consequently, an overestimation of their relative proportion in the 2-monoacyl-*sn*-glycerol moiety after pancreatic lipase action was expected (8,9,36). Indeed, the 22:6n-3 content of the *sn*-2 position obtained with the pancreatic lipase method was higher than the content obtained by calculation after Grignard degradation. Because the difference was close to significance ($P = 0.06$), and as one of our aims was to focus on VLC-PUFA distribution, we chose the Grignard degradation method in order to most accurately assess the positional distribution of the fatty acids.

The stereospecificity of phospholipase C and the completeness of the reaction induced by this enzyme were significant to selectively measure the distribution of fatty acids in the resulting 1,2-diacyl-*sn*-glycerols and in the 2,3-diacyl-*sn*-glycero-1-phosphocholines, which were then used to calculate the fatty acid content in the *sn*-1 and *sn*-3 positions.

The positional distribution of saturated, monounsaturated and 18-carbon essential fatty acids observed in the present study agrees with previous data (see Table 4). There are, however, minor discrepancies that may be accounted for in part by differences in the number of samples examined. Medium-chain saturated fatty acids showed a highly selective distribution in human colostrum and mature milk TAG. The proportion of saturated fatty acids increased in the *sn*-2 position and decreased in the *sn*-3 position as the carbon chain lengthened from 10 to 16 carbons. Such data indicate that the length of the carbon chain is a discriminating factor in the acylation process of medium-chain saturated fatty acids, especially to the *sn*-2 and *sn*-3 positions. However, this was not true for fatty acids with more than 16 carbons and/or with one or more double bonds as similar profiles were observed for 18:2n-6 and 18:3n-3, for 18:0 and 18:1, for 20:4n-6 and 22:6n-3 and for 22:4n-6 and 22:5n-3. Whether common biochemical pathways are responsible for the incorporation of these specific pairs of fatty acids into milk TAG or whether specific acyl transferases and/or a fatty acid binding protein (37) are involved in the stereospecific distribution of fatty acids in the glycerol molecule is unknown.

In humans, the stereospecific distribution of the TAG fatty acids differs between milk and adipose tissue (Table 4). This underlines the relative specificity of the mammary gland for the placement of the fatty acids in the three positions of milk TAG.

The similarities of the fatty acid positional distribution in colostrum and mature milk TAG indicate that the maturation of the mammary gland does not affect the acylation of fatty acids to the glycerol backbone.

About half of the 20:4n-6 and 22:6n-3 content of human milk TAG is found in the *sn*-2 position, the other half is esterified to the *sn*-3 position. From this positional distribution, one may surmise that a similar proportion of 20:4n-6 and 22:6n-3 is absorbed as 2-monoacyl-*sn*-glycerol and as free fatty acid by the newborn enterocytes after intestinal hydrolysis of ingested human milk TAG. This is likely to be the case in the breast-fed infant inasmuch as the resistance of the arachidonate and docosahexaenoate located at the outer positions to pancreatic lipase hydrolysis (8,9,36) is circumvented by gastric lipase and bile salt stimulated lipase (38–44). Indeed, it has been shown that in the small intestine of five-week-old dogs, freshly expressed milk TAGs are almost completely hydrolyzed by these three lipases mainly to yield free fatty acids and monoacylglycerols (45).

In contrast to human milk, infant formulas are devoid of any bile salt stimulated lipase activity, and a consequent reduction in hydrolysis of the *sn*-1 and *sn*-3 ester bonds of TAG may be expected. This limitation could be overcome if the source of TAG was adequately chosen to fortify formulas with VLC-PUFA in the *sn*-2 position of TAG.

Based on our results, the docosahexaenoic acid (DHA) status of infant fed formulas which are fortified only with a physiological amount of DHA equally distributed between the free and the 2-monoacyl-*sn*-glycerol form, would be worthy of further investigation.

In conclusion, the highly specific positional distribution of VLC-PUFA in human milk TAG is likely to be of biological significance. These findings could prove valuable in designing efficient lipid sources for infant formulas.

TABLE 3

Results (mol%; means ± SD) of the Stereospecific Analysis of Mature Milk Triacylglycerols (TAG) (n = 4)

Fatty acids	*sn*-1 (%)	*sn*-2 (%)	*sn*-3 (%)	Calculated TAG (%)[a]	Original TAG (%)
Saturated					
10:0	0.43 ± 0.91	0.62 ± 0.73	2.31 ± 1.31	1.12 ± 0.53	1.52 ± 0.64
12:0	2.31 ± 4.39	7.81 ± 4.29	13.90 ± 4.01	8.00 ± 4.01	7.75 ± 3.49
14:0	3.52 ± 2.20	12.45 ± 3.55	10.67 ± 4.01	8.88 ± 2.91	8.02 ± 2.27
15:0	0.22 ± 0.10	0.75 ± 0.20	0.41 ± 0.21	0.46 ± 0.10	0.42 ± 0.13
16:0	12.40 ± 4.14	51.22 ± 1.54	11.65 ± 6.25	25.09 ± 3.56	24.02 ± 1.21
17:0	0.43 ± 0.13	0.30 ± 0.09	0.22 ± 0.12	0.32 ± 0.09	0.32 ± 0.05
18:0	15.16 ± 2.05	1.45 ± 0.50	5.16 ± 1.28	7.26 ± 0.83	7.39 ± 0.29
20:0	0.15 ± 0.05	0.20 ± 0.03	0.00	0.10 ± 0.05	0.14 ± 0.02
Monounsaturated					
14:1n-5	0.28 ± 0.27	0.18 ± 0.06	0.73 ± 0.24	0.40 ± 0.16	0.22 ± 0.08
16:1[b,d]	1.59 ± 0.59	2.41 ± 0.31	3.20 ± 0.95	2.40 ± 0.23	2.16 ± 0.45
18:1[b,d]	46.38 ± 5.48	11.48 ± 5.19	31.77 ± 7.25	29.88 ± 5.70	31.24 ± 6.40
20:1[c]	0.47 ± 0.20	0.30 ± 0.15	0.34 ± 0.22	0.37 ± 0.18	0.47 ± 0.09
22:1n-9	0.29 ± 0.25	0.17 ± 0.17	0.21 ± 0.10	0.22 ± 0.10	0.10 ± 0.04
24:1n-9	0.00	0.00	0.00	0.00	0.00
n-6 Polyunsaturated					
18:2n-6[d]	14.64 ± 4.20	8.45 ± 0.98	16.70 ± 1.35	13.26 ± 2.05	13.83 ± 1.96
20:2n-6	0.37 ± 0.20	0.24 ± 0.25	0.27 ± 0.10	0.29 ± 0.14	0.30 ± 0.17
22:2n-6	0.12 ± 0.25	0.00	0.00	0.00	0.00
20:3n-6	0.23 ± 0.04	0.14 ± 0.04	0.22 ± 0.04	0.19 ± 0.04	0.22 ± 0.07
20:4n-6	0.05 ± 0.06	0.40 ± 0.12	0.37 ± 0.08	0.27 ± 0.05	0.30 ± 0.10
22:4n-6	0.00	0.14 ± 0.05	0.00	0.05 ± 0.05	0.05 ± 0.04
n-3 Polyunsaturated					
18:3n-3	0.89 ± 0.65	0.80 ± 0.76	1.41 ± 0.92	1.03 ± 0.77	1.08 ± 0.86
20:3n-3	0.00	0.00	0.00	0.00	0.00
18:4n-3	0.0	0.00	0.00	0.00	0.21 ± 0.19
20:4n-3	0.00	0.00	0.00	0.00	0.00
20:5n-3	0.00	0.00	0.00	0.00	0.00
22:5n-3	0.00	0.22 ± 0.03	0.00	0.07 ± 0.05	0.07 ± 0.06
22:6n-3	0.00	0.26 ± 0.05	0.13 ± 0.06	0.13 ± 0.03	0.15 ± 0.06

[a]{*sn*-1 + *sn*-2 + *sn*-3)/3 was used for the calculated triacylglycerols.
[b]n-7 + n-9 Isomers.
[c]n-9 + n-11 Isomers.
[d]Includes *trans* isomers.

TABLE 4

Preferential Distribution of Fatty Acids in the Triacylglycerols of Human Milk and Subcutaneous Adipose Tissue[a]

	n	Fatty acids										
		10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4
Milk fat												
Present study[b]	15	3,2,1	3,2,1	2,3,1	2,1,3	3,2,1	1,3,2	1,3,2	3,1,2	3,1,2	1,3,2	(2,3),1
Breckenridge[c]	3	3,1,2	3,2,1	2,3,1	2,1,3	3,2,1	1,2,3	3,1,2	3,1,2	3,2,1	1,2,3	2,3,1
Christie[d]	1	3,2,1	3,2,1	2,3,1	2,1,3	3,1,2	1,2,3	3,1,2	3,1,2			
Smith[e]	3	2-	2-	2-	2-	1,3-	1,3-	1,3-				
Tomarelli[f]	1	1,3-	2-	2-		1,3-	1,3-	1,3-	1,3-			
Adipose tissue												
Brockerhoff[g]	2			2,1,3	1,3,2	2,1,3	1,3,2	(3,2),1	2,3,1	(1,2,3)		

[a]For each fatty acid, the numbers denote the sites of esterification in glycerol (1, *sn*-1; 2, *sn*-2; 3, *sn*-3). The sequence of numbers indicates the distribution in a decreasing order. Numbers in parentheses denote sites occupied by a similar proportion of fatty acids.
[b,c,d,g]Stereospecific analysis ([c]Ref. 46; [d]Ref. 47; [g]Ref. 49).
[e,f]Pancreatic lipase analysis ([e]Ref. 48; [f]Ref. 14).

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Dietary Saturated, Monounsaturated, n-6 and n-3 Fatty Acids, and Cholesterol Influence Platelet Fatty Acids in the Exclusively Formula-Fed Piglet

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Platelet lipid composition is important to normal platelet morphology and function, and is influenced by dietary fatty acids and cholesterol. The fatty acid composition and cholesterol content of infant formulas differs from those of human milk, but the possible effects on platelet lipids in young infants is not known. This was studied in piglets fed from birth to 18 d of age with one of eight formulas differing in saturated fatty acid chain length, or content of 18:1, 20:5n-3 plus 22:6n-3, or cholesterol. A reference group of piglets fed sow milk was also studied. Sow milk has a fatty acid composition and cholesterol content similar to that of human milk. Piglets fed formulas high in 18:1 (34.9–40.8% wt fatty acids) and low in 16.0 (≤6.5% wt fatty acids) had lower platelet counts and greater platelet size than piglets fed sow milk (40.4% 18:1, 30.7% 16:0). Piglets fed formulas high in 16:0 (27–29.6%) and 18:1 (40–40.6%), or low in both 16:0 (5.9–6.1%) and 18:1 (10.8–11.2%), had similar platelet counts and size to piglets fed sow milk. Platelet phospholipid % 20:4n-6 was lower in all the groups of piglets fed formula than in the group fed sow milk. Addition of fish oil with 20:5n-3 plus 22:6n-3 to the formula further decreased platelet phospholipid 20:4n-6. Addition of cholesterol to the formula increased the platelet phospholipid % 20:4n-6 and platelet volume. *Lipids 28,* **645–650 (1993).**

Numerous studies in adult humans and other species have shown that dietary fat composition influences the fatty acid composition of platelet phospholipids and that changes in platelet phospholipid fatty acids may be accompanied by alterations in platelet function and platelet number (1–15). Diets containing marine animal fat or fish oil are known to increase platelet membrane levels of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) and decrease arachidonic acid (20:4n-6), alter platelet half-life, size and number, decrease aggregatory activity and increase bleeding time (2–7). Studies that show that dietary saturated fat, saturated to monounsaturated fatty acid balance and oleic acid (18:1) and linoleic acid (18:2n-6) content alter platelet phospholipid fatty acids, platelet number and/or platelet function have been published (1–5,8–12). High dietary intakes of cholesterol, on the other hand, have been found to increase platelet phospholipid 20:4n-6 levels (8,13) and sensitivity to some aggregatory agonists (13–15).

Fat represents about 45–50% of the energy in human milk and infant formulas, and milk or formulas often provide the sole source of nutrition for infants for at least three months after birth. The fatty acid composition and cholesterol content of infant formulas usually differs from that of human milk (16). Human milk fatty acids usually contain 20–25% palmitic acid (16:0), 32–38% oleic acid (18:1), 7–16% linoleic acid (18:2n-6), small amounts of linolenic acid (18:3n-3), 20:4n-6 and 22:6n-3 and about 20 mg cholesterol/dL milk (16,17). Infant formulas often contain much lower amounts of 16:0 and 18:1, higher 18:2n-6 and little or no 20:4n-6, 22:6n-3 or cholesterol. Formulas for preterm and other infants who may have low bile salt pools and/or lipase activity often contain medium-chain fatty acids (8:0 plus 10:0), whereas formulas for term infants often contain 12:0 and 14:0, rather than 16:0, as the major source of saturated fatty acids. Some formulas contain 16:0, but the positional distribution of fatty acids in the triglycerides differs from that in milk fats (16,17). Fish oils have recently been added to some preterm infant formulas to provide a dietary source of 22:6n-3 (18,19). The information published on the effects of dietary fat on platelet lipid composition, size and number in adult humans and other species suggests that the differences in fat composition among formulas and milk may result in differences in platelet lipid composition, possibly in platelet size and number and in infants. The effects of formula feeding on platelet lipids or morphology in human infants has not been reported and is difficult to study because of practical limits in blood sample size.

Pig milk has a lipid composition similar to that of human milk, and pathways of lipid digestion, absorption and metabolism are also similar in piglets and in human infants (20,21). The objective of the present studies was to determine the effect of the saturated fatty acid chain length (8:0 plus 10:0, 12:0 plus 14:0, or 16:0), 18:1 content, addition of 20:5n-3 and 22:6n-3 from fish oil, and cholesterol content of formula on platelet phospholipid fatty acid composition, platelet size and number in piglets fed with formulas from birth. For practical reasons related to the number of samples studied, and difficulty of extrapolating results from *in vitro* tests of platelet function to physiological events *in vivo*, tests of platelet aggregatory activity were not included.

MATERIALS AND METHODS

Animals and diets. Male Yorkshire piglets of term gestation were taken within 12 h of birth and randomly assigned to one of eight formula groups. Piglets fed sow milk were identified at birth, but left with their natural mothers. Animals within a dietary group were not littermates. Piglets were fed the formulas by bottle every 3–4 h from 0700 to 2400 h until the eighteenth day after birth. Passive immunity was provided by addition of pig serum-derived immunoglobulin to the formula for the first 72 h (20).

Eight formulas, identical in all respects except for fat composition, were designed as two groups, four formulas each, as shown in Table 1 and 2. The formulas in Group 1 had similar amounts of 18:2n-6 and 18:3n-3, and all

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Abbreviations: APTT, activated partial thromboplastin time; GLC, gas-liquid chromatography; MCT, medium-chain triglycerides; PDW, platelet distribution width; PT, prothrombin time.

TABLE 1

Oil Blends of Formulas

Fat blend (% total fat by vol)	Group 1				Group 2			
	A	B	C	D	E	F	G	H
MCT[a]	40	40	40	20	31			
Coconut	20	20	20	10		34.7	7	7
Soybean	40	37	40	40				
High oleic[b]				30		26.1	8.9	9
Canola					33.3	33.3	33.1	
Palm							42.4	
Safflower					9.6	8.8	8.6	3.2
Betapol								
Menhaden		3						
Cholesterol (mg/dl)			42					

[a]MCT, medium-chain triglycerides.
[b]High oleic safflower or sunflower oil.

contained a low amount of 16:0 (Table 2). In three of the formulas, 40% of the fat blend was medium-chain triglyceride oil (MCT, 42.5 wt% formula fatty acids as 8:0 plus 10:0) with 10.8–11.2% 18:1; one formula had no additions (A), one had 3% fat blend as fish oil [0.5 and 0.3 wt% formula fatty acids as 20:5n-3 and 22:6n-3, respectively (B)] and one had 42 mg cholesterol (10 mg free cholesterol, 32 mg cholesteryl palmitate)/dL (C) (Table 1). The other formula in Group 1 had 20% of the formula fat blend as MCT (21.2 wt% formula fatty acids as 8:0 plus 10:0) with 18:1 increased to 34.9% 18:1 (D) (Table 2). The formulas in Group 2 contained similar amounts of 18:1, 18:2n-6 and 18:3n-3 but differed in the chainlength of the predominant saturated fatty acids; 8:0 plus 10:0 (E), 10:0 plus 12:0 (F) or 16:0 (G and H). Formula G had 27.0% 16:0, largely from palm oil (Table 2), with 6.5% 16:0 esterified to the 2-position of the triglyceride glycerol (22). Formula H contained Betapol, a synthetic triglyceride which had 29.6% 16:0, of which about 70% was esterified to the 2-position of the triglyceride glycerol (22), as it is in human (17) and in pig (22) milk. Formula H was included because previous studies found that the position of 16:0 in formula triglycerides influenced the fatty acid composition of plasma lipids in formula-fed piglets (22). All procedures used in these studies were approved by the University of British Columbia Animal Care Committee and conformed to guidelines set by the Canadian Council on Animal Care.

Analytical procedures. Blood was collected (20) from anesthetized piglets (Ketamine, MTC Pharmaceuticals, Cambridge, Ontario, Canada; plus Rompun, Bayvet Division, Chemagro Ltd. Etobicoke, Ontario, Canada; 37.5 mg/kg plus 3.75 mg/kg, respectively) after an overnight fast. The first 10 mL blood collected was not used for platelet assays. Platelet-rich plasma was prepared from blood by centrifugation for 15 min at 120 × *g* at 4°C. The platelets were recovered by further centrifugation, 15 min at 1100 × *g* at 4°C, using plastic tubes. The platelets were washed twice with 3 mM ethylenediaminetetraacetic acid in saline. Plasma was separated by centrifugation of whole blood at 2500 × *g* for 15 min at 4°C. Samples for lipid analyses were stored at −70°C. Measurements of platelet count, volume and platelet distribution width (PDW, a measure of the variation in platelet volume) and of prothrombin time (PT) and activated partial thromboplastin time (APTT) were done by routine procedures used in the Hematopathology Laboratory of the B.C. Children's Hospital, employing an automated multiparameter blood counter TOA Sysinex NE-8000, NE-5500 (CanLab, Vancouver, British Columbia, Canada).

Plasma and platelet lipids were extracted, and phospholipids, triglycerides and cholesteryl esters were separated by thin-layer chromatography and recovered (20). Platelet and plasma phospholipid fatty acids were converted to their respective methyl esters with methanolic HCl (1:5, vol/vol) at 100°C for 5 min. Methyl esters of fatty acids in plasma triglycerides and cholesteryl esters were prepared by reaction with BF_3/benzene/methanol (25:20:55, by vol) at 100°C for 30 min and BF_3/benzene/methanol (35:30:35, by vol) at 100°C for 45 min, respectively. Methyl esters were recovered, separated and quantitated by capillary column gas-liquid chromatography (GLC) with 17:0 as the internal standards (20). The fatty acid composition of plasma lipids from these piglets has been published (22).

TABLE 2

Major Fatty Acid Components[a]

Fatty acids (% total fatty acids by wt)	Formula								Pig milk
	Group 1				Group 2				
	A	B	C	D	E	F	G	H	
8:0	30.5	30.5	30.5	15.2	22.2	3.2	0.5	0.6	0.1
10:0	12.0	12.0	12.0	6.0	8.2	1.9	0.4	0.4	0.2
12:0	9.4	9.4	9.4	4.7	0.6	19.1	3.3	3.3	0.2
14:0	3.6	3.9	3.6	1.8	0.1	6.8	2.1	1.8	3.1
16:0	5.9	6.1	5.9	6.0	4.3	6.5	27.0	29.6	30.7
18:0	2.4	2.3	2.4	3.7	3.0	3.4	5.6	3.1	4.4
18:1	11.2	10.8	11.2	34.9	40.8	39.0	40.0	40.6	40.4
18:2n-6	21.7	20.2	21.7	23.7	16.2	15.6	16.4	16.4	8.2
18:3n-3	3.1	2.9	3.1	3.2	3.4	3.2	3.1	3.2	0.8
20:1					0.5	0.4	0.4	0.4	0.1
20:5n-3		0.5							0.2
22:6n-3		0.3							0.1

[a]The formulas contained no C_{20} or C_{22} ± n-6 fatty acids, the sow milk had 0.7% 20:4n-6. Levels of 22:1 did not exceed 0.1% fatty acids in any formula.

The cholesterol content in extracted platelet lipid was determined with enzymatic kit reagents (BioPacific Diagnostic Inc., North Vancouver, British Columbia, Canada). Phospholipid content was calculated from the quantitative analyses of phospholipid fatty acids by GLC. Phospholipid fatty acid unsaturation indices (UIs) were calculated as described previously (23).

Statistical analysis. Differences between a group of formula-fed and the sow milk-fed piglets, differences between piglets fed Formula B, C or D and piglets fed Formula A, and differences among the groups fed Formulas E, F, G and H were determined using two-way analysis of variance. The number of animals in each group was six, except for the Formula G with saturated fatty acids from palm oil, in which there were five piglets. The statistical analyses were performed with the Number Cruncher Statistical System, version 5.01 (Kaysville, UT).

RESULTS

Platelet number and size. Considerable variability in platelet numbers, PDW and volume was found among the piglets (Table 3). Similar variability in platelet size and numbers also occurs within and among healthy humans (24). Piglets fed Formula D with 20% MCT plus 34.9% 18:1 had significantly lower platelet numbers and higher platelet PDW and volumes than piglets fed sow milk, and lower platelet numbers than piglets fed Formula A with 40% MCT plus 11.2% 18:1 (Table 2). Piglets fed Formulas E and F with high 18:1 and saturated fatty acids as 8:0 plus 10:0, or 12:0 plus 14:0, also had significantly lower platelet numbers and higher platelet PDW and volumes than piglets fed sow milk or piglets fed Formulas G and H with high 18:1 and saturated fatty acids as 16:0. The three formulas (D, E and F) associated with reduced platelet number and increased platelet size differed from sow milk and Formulas A, B, C, G and H in that they contained high 18:1 (34.9 to 40.8% fatty acids) but low 16:0 (4.3 to 6.5% fatty acids) (Table 2).

Piglets fed Formula C with 40% MCT and cholesterol had a significantly higher mean platelet volume than piglets fed sow milk; the value, however, was not significantly different from that of piglets fed the same formula without cholesterol (Formula A). Addition of n-3 fatty acids from fish oil to the formula had no significant effect on platelet number or size. No significant differences in PT or APTT were found among the groups of formula- and sow milk-fed piglets.

Platelet phospholipid and cholesterol. The platelet lipid cholesterol/phospholipid (mol/mol) ratio was significantly higher in piglets fed Formula A, B, E, F, G or H than in piglets fed sow milk (Table 4). Piglets fed Formula C with cholesterol, or Formula D with low 16:0 and high 18:1 had lower cholesterol/phospholipid ratios in platelet lipid than piglets fed Formula A with low 16:0 and low 18:1, and no added cholesterol. No significant statistical relationships were found between the plasma cholesterol concentration (data not shown) and the platelet lipid cholesterol/phospholipid ratio.

Platelet phospholipid fatty acids. Formulas A, B, C and D contained lower levels of 16:0 (5.9–6.1% fatty acids) and higher 18:2n-6 (20–24%) and 18:3n-3 (2.9–3.2%) than the sow milk (Table 2). The platelet phospholipids of piglets fed these formulas had a significantly lower percentage of 16:0 and 20:4n-6 and significantly higher 18:0 and 18:2n-6 than piglets fed sow milk (Table 4). The platelet phospholipid 18:1 percentage was significantly lower in piglets fed Formulas A, B and C containing 10.8–11.2%, than in piglets fed Formula D with 34.9% 18:1, or piglets fed sow milk. The 18:1 percentage in platelet phos-

TABLE 3

Platelet Counts and Size and Clotting Factor Indices in Piglets Fed Formulas of Varying Fat Composition or Sow Milk[a]

Diet group	Platelet count ($\times 10^9$/L)	Mean platelet diameter (fl)	Mean platelet volume (fl)	Prothrombin time (s)	Partial thromboplastin time (s)
Sow milk	691 ± 150	11.4 ± 0.7	9.0 ± 0.2	12.1 ± 0.1	14.0 ± 0.6
Group 1 formulas					
A	617 ± 45	12.2 ± 0.8	10.0 ± 0.4	11.9 ± 0.1	14.1 ± 0.7
B	596 ± 124	12.2 ± 0.8	9.8 ± 0.4	12.3 ± 0.2	14.1 ± 0.6
C	558 ± 55	12.8 ± 0.6	10.1 ± 0.3[c]	12.7 ± 0.4	13.9 ± 3.6
D	474 ± 30[b,d]	13.3 ± 0.5[b]	10.2 ± 0.2[c]	12.2 ± 0.2	14.5 ± 1.2
Group 2 formulas					
E	500 ± 35[b]	14.7 ± 0.8[c]	10.8 ± 0.4[c]	11.8 ± 0.4	13.6 ± 1.5
F	436 ± 17[c]	15.5 ± 1.0[c]	10.9 ± 0.5[c]	11.9 ± 0.2	15.5 ± 0.5
G	684 ± 45[e]	12.4 ± 0.6	9.9 ± 0.3	12.6 ± 0.8	15.0 ± 0.5
H	628 ± 70	12.0 ± 0.7[f]	9.5 ± 0.3[f]	11.9 ± 0.3	13.7 ± 0.2

[a]Values are means ± SEM for piglets. The formulas are described in Tables 1 and 2, fl, femtoliters.

[b,c]The P value for the difference between piglets fed a given formula and piglets fed sow milk are [b]$P < 0.05$ and [c]$P < 0.0125$, respectively.

[d]Value for piglets fed Formula D significantly different from value for piglets fed Formula A, $P = 0.03$.

[e,f]Value for piglets fed Formula G or H, respectively, significantly different from value for piglets fed Formula F, $P < 0.05$.

TABLE 4

Distribution of Major Fatty Acids in Platelet Phospholipids and Platelet Lipid Cholesterol/Phospholipid Ratio of Piglets Fed Formulas Varying in Fat Composition or Sow Milk[a]

	Sow milk	Group 1				Group 2			
		A	B	C	D	E	F	G	H
14:0	0.2 ± 0.0	0.4 ± 0.0[b]	0.3 ± 0.0	0.2 ± 0.0[e]	0.2 ± 0.0[d]	0.2 ± 0.0[f]	0.6 ± 0.1[c,g]	0.1 ± 0.0[f]	0.1 ± 0.0[b,f]
16:0	23.7 ± 0.5	17.7 ± 0.8[c]	16.2 ± 0.3[c]	15.1 ± 0.4[c,d]	16.7 ± 0.2[c]	15.7 ± 0.7[c,f]	17.5 ± 0.3[c,f,g]	18.5 ± 1.0[c,g,h]	20.4 ± 0.4[h]
18:0	15.4 ± 0.3	24.1 ± 0.5[c]	25.8 ± 0.3[c,d]	24.5 ± 0.2[c]	22.4 ± 0.3[c,d]	21.0 ± 0.4[c,f]	18.8 ± 0.2[c,i]	16.5 ± 0.5[g]	15.1 ± 0.2[h]
Σ Sat	40.3 ± 0.6	43.6 ± 0.6[c]	43.0 ± 0.2[b]	40.5 ± 0.5[d]	40.0 ± 0.2[e]	37.6 ± 0.6[c]	37.8 ± 0.3[c]	36.1 ± 0.6[c]	36.1 ± 0.3[c]
18:1	22.7 ± 0.2	16.3 ± 0.5[c]	16.5 ± 0.3[c]	16.5 ± 0.7[c]	25.2 ± 0.5[b,e]	31.7 ± 0.4[c,f]	29.3 ± 0.3[c,h]	26.4 ± 0.4[c,g]	26.4 ± 0.4[c,g]
Σ Mono	24.1 ± 0.2	17.0 ± 0.5[c]	16.9 ± 0.3[c]	17.1 ± 0.7[c]	26.1 ± 0.4[e]	33.4 ± 0.4[c,f]	30.4 ± 0.3[c,h]	27.4 ± 0.4[c,g]	27.3 ± 0.4[c,g]
18:2n-6	5.9 ± 0.4	14.4 ± 0.7[c]	16.6 ± 0.5[c]	14.9 ± 0.4[c]	12.1 ± 0.4[c,d]	9.9 ± 0.3[c]	9.6 ± 0.4[c]	9.2 ± 0.5[c]	10.2 ± 0.3[c]
20:3n-6	1.0 ± 0.1	1.4 ± 0.1	1.4 ± 0.2	1.4 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
20:4n-6	23.4 ± 0.2	17.8 ± 0.8[c]	15.0 ± 0.5[c,d]	20.1 ± 0.4[b,d]	16.0 ± 0.5[c]	14.9 ± 0.4[c,f]	16.7 ± 0.3[c,h]	20.9 ± 0.3[b,g]	20.1 ± 0.2[c,g]
22:4n-6	2.2 ± 0.1	2.5 ± 0.3	1.7 ± 0.3[d]	2.3 ± 0.2	2.3 ± 0.2	1.1 ± 0.1[c,f]	1.3 ± 0.1[c,f,g]	1.6 ± 0.1[b,g]	1.4 ± 0.1[c,g]
22:5n-6	0.3 ± 0.0	0.1 ± 0.0[c]	0.1 ± 0.0[c,e]	0.2 ± 0.0[c]	0.1 ± 0.0[c]	trace[c]	0.1 ± 0.0[c]	0.1 ± 0.0[c]	0.1 ± 0.0[c]
Σ n-6	33.1 ± 0.4	37.5 ± 0.5[c]	35.8 ± 0.2[c,d]	40.2 ± 0.5[c,d]	32.4 ± 0.3[e]	27.0 ± 0.8[c,f]	29.6 ± 0.4[h]	33.4 ± 0.5[g]	33.6 ± 0.4[g]
20:5n-3	0.4 ± 0.0	0.4 ± 0.0	2.2 ± 0.1[c,e]	0.5 ± 0.0	0.3 ± 0.0[d]	0.5 ± 0.0[f]	0.6 ± 0.0[c,f,h]	0.7 ± 0.1[c,g,h]	0.8 ± 0.1[c,g]
22:5n-3	1.3 ± 0.0	0.9 ± 0.1[c]	1.2 ± 0.1[d]	0.9 ± 0.1[c]	0.6 ± 0.1[c,d]	0.7 ± 0.1[c,f]	0.7 ± 0.0[f]	1.2 ± 0.1[g]	1.1 ± 0.1[g]
22:6n-3	0.6 ± 0.0	0.4 ± 0.1	0.8 ± 0.1[d]	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0[f]	0.5 ± 0.0[f]	0.9 ± 0.1[g]	0.7 ± 0.0[g]
Σ n-3	2.5 ± 0.1	2.0 ± 0.1[b]	4.4 ± 0.2[c,e]	2.1 ± 0.2	1.6 ± 0.1[c,d]	2.0 ± 0.0[f]	2.2 ± 0.1[f]	3.2 ± 0.2[c,g]	3.0 ± 0.1[g]
C_{20} + C_{22} n-6/n-3	11.7 ± 0.4	13.6 ± 0.8	4.7 ± 0.3[c,e]	13.9 ± 0.8	15.5 ± 0.8[b]	10.5 ± 0.7[f,g]	11.0 ± 0.4[f]	8.7 ± 0.5[c,g]	8.8 ± 0.3[c,g]
UI	156 ± 1	144 ± 3[b]	146 ± 1[b]	155 ± 2[d]	137 ± 1[c,d]	129 ± 3[c,f]	138 ± 1[c,h]	156 ± 2[g]	154 ± 1[g]
Cholesterol/ phospholipid	0.60 ± 0.1	0.73 ± 0.03[b]	0.67 ± 0.03	0.68 ± 0.01[d]	0.66 ± 0.01[d]	0.78 ± 0.01[e,f,g]	0.70 ± 0.01[b,f]	0.74 ± 0.02[f,g]	0.85 ± 0.02[c,g]

[a]Values given are means ± SEM; fatty acids (% wt total); cholesterol/phospholipid (mol/mol). The composition of the formulas is described in Tables 1 and 2. Trace indicates value <0.1% fatty acids; Σ, sum; UI, fatty acid unsaturation index. Fatty acids C<14:0 and 18:3n-3 represented less than 0.1% fatty acids in all groups.
[b,c]Values for piglets fed formula which are significantly different from values for piglets fed sow milk are indicated as [b]$P < 0.05$, [c]$P < 0.0125$. [d,e]Values for piglets fed formula B, C or D were compared to values for piglets fed formula A; a superscript[d] or [e] indicates value significantly different from the value for piglets fed Formula A, $P < 0.05$ or $P < 0.0125$, respectively. [f,g,h,i]Values for piglets fed Formulas E, F, G and H were compared to each other; values with a different superscript are significantly different, $P < 0.05$.

pholipids from piglets fed Formula D, however, was higher than in piglets fed the sow milk containing 40.4% 18:1. Although Formulas A and D had similar amounts of 18:0, 18:2n-6 and 18:3n-3, the platelet phospholipid % 18:0, 18:2n-6 and 22:5n-3, and the phospholipid fatty acid UI was significantly lower in piglets fed Formula D than in piglets fed Formula A.

Piglets fed Formula B with fish oil had significantly higher platelet phospholipid 20:5n-3, 22:5n-3 and 22:6n-3 and significantly lower % 20:4n-6, 22:4n-6 and 22:5n-6 than piglets fed Formula A without fish oil (Table 4). The C_{20} plus C_{22} n-6 to n-3 fatty acid ratio was 4.7 ± 0.3 in piglets fed Formula B, compared to 11.7 ± 0.4 in piglets fed sow milk and mean values of 13.6–15.5 in piglets fed Formulas A, C and D (Table 4). The UI, however, was similar in piglets fed Formula A and B, and significantly lower than in piglets fed sow milk.

Although the fatty acid composition of Formulas A and C were similar, piglets fed Formula C with cholesterol had a significantly lower % 16:0 and higher % 20:4n-6 in platelet phospholipids than piglets fed Formula A without cholesterol. The UI value of piglets fed Formula C was also significantly increased when compared to piglets fed Formula A, but it was not different from that of piglets fed sow milk. Piglets fed Formulas E, F, G and H with 39–40.8% 18:1 and 15.6–16.4% 18:2n-6 had a significantly higher % 18:1 and 18:2n-6 and a significantly lower % 20:4n-6, 22:4n-6 and 22:5n-6 in their platelet phospholipids than piglets fed sow milk (Table 4). The % 16:0 and 18:0 in the phospholipid was significantly, and inversely, altered by the saturated fatty acid composition of the formula fed. Piglets fed Formulas E or F containing 8:0 plus 10:0 or 12:0 plus 14:0 had significantly higher 18:0 and lower 16:0 in platelet phospholipids than in piglets fed sow milk or Formulas G or H with 16:0. The % 18:1, 20:4n-6, 22:5n-3 and 22:6n-3 in platelet phospholipids was also influenced by the saturated fatty acid composition of the formula. Piglets fed Formulas G or H with 27–29.6% 16:0 had a significantly lower % 18:1 and higher % 20:4n-6, 22:5n-3 and 22:6n-3 in platelet phospholipids than piglets fed Formulas E or F with 8:0 plus 10:0 or 12:0 plus 14:0 (Table 4). The % 18:1 and 18:2n-6 was significantly higher, and % 20:4n-6, 22:4n-6 and 22:5n-6 was significantly lower in platelet phospholipids of piglets fed Formulas E, F, G and H than in piglets fed sow milk. The platelet phospholipid % 20:5n-3 in piglets fed Formula F, G or H and the % 22:6n-3 in piglets fed Formula G was significantly higher, but the % 22:5n-3 in piglets fed Formula E was lower than in piglets fed sow milk. The platelet phospholipid UI was similar among piglets fed sow milk and Formulas G or H, but piglets fed Formulas E or F had a significantly lower UI than in piglets fed sow milk. The difference in the position of 16:0 in the triglycerides of Formulas G and H had no apparent effect on the fatty acid composition of the platelet phospholipids.

DISCUSSION

The results of these studies show that the fat composition of the formula fed to young piglets influences the lipid composition, number and size of platelets. Diet-related changes in platelet phospholipid fatty acids could occur during *de novo* phospholipid synthesis, or later during exchange of intact phospholipids with plasma lipoprotein

phospholipids, or by phospholipid deacylation and reacylation (25–27). The fatty acid composition of human platelet phospholipid is known to show a high degree of correspondence with plasma phospholipid, triglyceride and cholesteryl ester fatty acids (28). The piglet platelet phospholipid % 16:0, 18:1 and 18:2n-6 was significantly ($P < 0.001$, n = 53 piglets) correlated with the % 16:0, 18:1 and 18:2n-6 in plasma phospholipids, r = 0.53, 0.95, 0.85; triglycerides, r = 0.70, 0.95, 0.91; and cholesteryl esters, r = 0.78, 0.91, 0.89, respectively. The platelet phospholipid % 20:4n-6 showed a significant correlation to the % 20:4n-6 in plasma phospholipid (r = 0.46, $P < 0.01$), but not in triglyceride or cholesteryl ester fatty acids. These relationships suggest that changes in platelet phospholipid fatty acid composition should be considered in infants known to have altered plasma lipid fatty acid compositions due to formula rather than breast-feeding.

Significant inverse correlations between the platelet phospholipid % 16:0 and 18:0, r = −0.71 $P < 0.001$; 18:1 and 18:2n-6, r = −0.70, $P < 0.001$; and 18:2n-6 and 20:4n-6, r = −0.40 $P < 0.05$; and a positive correlation between the % 16:0 and 20:4n-6, r = 0.51, $P < 0.001$, were also found. Palmitic acid (16:0) and 18:0 are usually esterified to the 1-position, whereas most of the 18:1, and almost all of the 18:2n-6 and 20:4n-6, is usually esterified to the 2-position of platelet phospholipids (26,29,30). An inverse relationship between 16:0 and 18:0 is apparent in other studies on the effects of dietary fat on human and animal platelet phospholipid fatty acids (5,10). As particular saturated fatty acids are preferentially combined with certain unsaturated fatty acids (30), it seems possible that the changes in 16:0 and 18:0 may have been secondary to diet-induced changes in 18:1, 18:2n-6 or 20:4n-6 at the 2-position of the platelet phospholipids. The higher % 18:2n-6 in the platelet phospholipids of piglets fed the formulas than in piglets fed sow milk was consistently accompanied by a lower % 20:4n-6. Competition between 18:2n-6 and 20:4n-6 for acylation into phospholipids has been suggested (31), and could be the result of the higher amounts of 18:2n-6 in the formulas than in the sow milk (Table 2). Lower platelet phospholipid 20:4n-6 has been reported for animals fed diets high in 18:2n-6 rather than saturated fat (8), and vegetable oils high in 18:1 or 18:2n-6 have been found to decrease platelet phospholipid 20:4n-6 in humans (1,10,11). The small amount of 20:4n-6 in sow milk, but absence of 20:4n-6 in the formula, could also explain the lower platelet 20:4n-6 in piglets fed formula rather than milk.

The increased % 20:4n-6 in the platelet phospholipid of piglets fed the formula with cholesterol (Table 4) is consistent with published data on the effects of dietary cholesterol on platelet cholesterol and 20:4n-6 in other species (8,13). The increase in platelet phospholipid 20:4n-6 was not accompanied by increased plasma phospholipid 20:4n-6 (6.6 ± 0.4% and 6.9 ± 0.4%, piglets fed Formula A with cholesterol and Formula C without cholesterol, respectively). Whether cholesterol as a component of natural milk is related to high platelet phospholipid % 20:4n-6 has not been considered.

Platelet phospholipid levels of 20:5n-3 are increased and 20:4n-6 is decreased in humans and in other animals fed diets containing marine lipid (2–7). Formula B with fish oil contained only 0.5% formula fatty acids as 20:5n-3, but still resulted in a significant decrease in the % 20:4n-6 in the platelet phospholipids of piglets fed the product (Table 4). Premature infants fed with formula have low plasma phospholipid levels of 20:4n-6, and 20:4n-6 is further reduced when these infants are fed formula with fish oil (18). The % 20:5n-3 in the platelet phospholipids of piglets fed Formula B was much higher than the % 22:6n-3 (2.2 ± 0.1, 0.8 ± 0.1% 20:5n-3 and 22:6n-3, respectively). Liver and brain of piglets fed similar formulas with fish oil, in contrast, contained only small amounts of 20:5n-3, and 22:6n-3 was present in much higher amounts (32). Evidence that 20:5n-3 may be incorporated into platelet phospholipids in higher amounts than 22:6n-3 has been published for humans consuming marine oils (3,7) and rats fed fish oil (5). The activity of rat platelet 1-acylglycerophosphocholine acyltransferase has been reported to be higher with 20:5n-3 than with 22:6n-3 as the substrate (33). Such enzyme substrate selectively could explain the apparent greater propensity of platelets than other cells to accumulate dietary 20:5n-3.

Liberation of 20:4n-6 from platelet phospholipid and subsequent oxygenation to proaggregatory eicosanoids is part of the mechanism involved in platelet reactivity (26,34,35). Although the availability of 20:4n-6 is a major determinant of platelet reactivity, this can be modified by other fatty acids, such as 20:5n-3, or fatty acid metabolites which compete or antagonize 20:4n-6 or its products. Changes in membrane cholesterol or phospholipid can also influence the response to stimuli by altering the physical properties of the membrane structure. Decreased platelet sensitivity to aggregating agonists has been found after feeding diets high in marine lipids (6,7), 18:2n-6 (9,10,12) or 18:1 (1,11), which also decreased platelet 20:4n-6. Oral administration of 20:4n-6 and diets high in saturated fat or cholesterol, on the other hand, have been found to increase platelet aggregability and 20:4n-6 (7,10,15,25,36).

Platelet volume is known to vary inversely, although not linearly, with platelet number in adult humans (24). The inverse changes in platelet number and size found in piglets in response to the different formula diets is, therefore, not unusual. The relationship of changes in platelet numbers and size to thrombopoiesis, and/or destruction, however, is not well understood. Decreased platelet numbers accompanied by increased platelet size was found in these studies in piglets fed formulas high in 18:1 (34.9–40.8%) and low in 16:0 (4.3–6.3%), but not in piglets fed formulas high in both 18:1 and 16:0 (27.0–29.6%), or low in both 16:0 (5.9–6.1%) and 18:1 (10.8–11.2%). Reduced platelet number and increased platelet size has been associated with reduced responsiveness to some aggregating agonists and lower platelet lipid 20:4n-6 (6,10,37). The decrease in platelet number has been suggested to possibly reflect decreased reactivity of platelets low in 20:4n-6, leading to reduced thrombopoiesis by reason of decreased thrombopoietin (37). Whether or not the lower platelet phospholipid 20:4n-6 in piglets fed formula rather than milk was accompanied by any change in platelet activity, survival rates, or thrombopoiesis is not known.

Our studies with piglets fed formulas with fat blends similar to those in infant formulas emphasize the need to consider the possible effects of formula feeding on platelet lipid composition and, more importantly, platelet size, number and function in human infants. The statistical

relationships between plasma and platelet lipid fatty acid composition may be of value in predicting possible groups at risk for diet-related changes in platelet composition. The possible sensitivity of platelet phospholipids to accumulate 20:5n-3 should be considered in the choice of oils used to provide a source of 22:6n-3 for infant formulas.

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Rat Vitamin E Status and Heart Lipid Peroxidation: Effect of Dietary α-Linolenic Acid and Marine n-3 Fatty Acids

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Three groups of sixteen male rats each were fed semipurified diets containing 15% by weight of lipid for a period of 4 wk. The diets contained the same amount of polyunsaturated fatty acids (PUFA) (20% of total fatty acids) and saturated fatty acids (19% of total fatty acids). Dietary PUFA were represented exclusively by linoleic acid (18:2 diet), or 10% linoleic acid and 10% linolenic acid (18:3 diet), or 10% linoleic acid and 10% long-chain n-3 fatty acids (LCn-3 diet). The overall amount of vitamin E was similar in the three diets, *i.e.*, 140, 133 and 129 mg/kg diet, respectively. Following appropriate extraction, tocopherol levels in heart, liver, brain, adipose tissue (AT) and plasma were measured by high-performance liquid chromatography. The level of vitamin E in the heart decreased with n-3 PUFA diets, most markedly with LCn-3 PUFA. Liver and AT vitamin E contents also decreased with n-3 PUFA diets when expressed as μg/mg total lipids and μg/mg phospholipids, respectively. Total plasma vitamin E was lower in rats fed the LCn-3 diet, but there was no significant difference when expressed as μg/mg total lipids. Brain vitamin E was not affected by the various diets. *In vitro* cardiac lipid peroxidation was quantified by the thiobarbituric acid reactive substances (TBARS) test. Heart homogenates were incubated at 37°C for 15 and 30 min in both the absence (uninduced) or presence (induced) of a free radical generating system (1 mM xanthine, 0.1 IU per mL xanthine oxidase, 0.2 mM/0.4 mM Fe/ethylenediaminetetraacetic acid). TBARS release was time-independent but significantly higher when LCn-3 fatty acids were fed to rats in either the uninduced or induced system. The study demonstrated that n-3 PUFA diets can influence vitamin E status of rats even in short-term experiments and can change the susceptibility of the heart to *in vitro* lipid peroxidation.
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Polyunsaturated fatty acids (PUFA) of vegetable or marine origin have been recommended to lower the incidence of coronary heart disease. Epidemiological studies in particular have shown an apparent beneficial effect of marine n-3 PUFA intake in reducing mortality from heart disease (1,2). Fish oils contain the longest chain n-3 PUFA, namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). These PUFA have five and six double bonds, respectively, and when they are present in the diet, they are readily incorporated into membrane phospholipids (PL), mainly at the expense of arachidonic acid (20:4n-6) (3). The result is an increase in the unsaturation of the membranes, making them more susceptible to peroxidation *in vitro* and *in vivo* (4–6). The major lipid-soluble chain-breaking antioxidant in plasma, red cells and tissues is vitamin E (7). The most potent antioxidant and biological form of the vitamin is α-tocopherol which scavenges peroxyl radicals by donating its phenolic hydrogen to them. Although several studies of feeding fish oils on the vitamin E status of animals have been carried out, few have compared diets providing the same amount of PUFA (5,6,8).

In the present study, rats were fed diets containing not only the same amount of PUFA from the n-6 and/or n-3 families [20% of the total fatty acids (FA)] but also the same amount of saturated FA. Dietary PUFA were represented exclusively by linoleic acid (18:2 diet), by 10% linoleic acid and 10% linolenic acid (18:3 diet), or by 10% linoleic acid and 10% long chain n-3 fatty acids (LCn-3 diet). The vitamin E status of the rats was measured, and the susceptibility to induced or uninduced heart lipid peroxidation conferred by n-6 or n-3 PUFA was determined.

MATERIALS AND METHODS

Animals and diets. Male specific pathogen-free rats of the Sprague-Dawley (Iffa Credo, L'Arbresle, France) strain were used. The animals weighed 80–100 g at the start of the experiment and were housed in individual stainless steel cages in a room of controlled temperature (21 ± 1°C), humidity (55–60%) and lighting (12 h dark-light cycle). The animals were allowed free access to food and sterile water. The rats were maintained on a commercial nonpurified diet (Usine d'Alimentation Rationnelle, Villemoisson-sur-Orge, France) for 5 d before transfer on the experimental diets (9). Three groups of sixteen animals each were fed semipurified diets for 4 wk. The diets contained 15% by weight of fat varying in the n-6 and n-3 PUFA content and type, but the total amount of PUFA was kept constant (20% of total FA). The 18:2 diet contained small amounts of n-3 PUFA (0.4% of total FA), the 18:3 diet contained linolenic acid as the only source of n-3 PUFA (10% of total FA), the LCn-3 diet contained the same amount of LCn-3 C_{20} and C_{22} PUFA (10% of the total FA). Saturated FA content was similar in all diets (19% of total FA). Various mixtures of refined vegetable oils (olive, palm, sunflower, linseed) and menhaden oil were used to obtain the appropriate fatty acid composition of the dietary fats (Table 1). The overall amount of vitamin E was high and similar in the three diets, *i.e.*, 140, 133 and 129 mg/kg diet for 18:2, 18:3 and LCn-3 diets, respectively. Thus, these levels were well above the 50 mg/kg vitamin E normally recommended for rat diets (10,11). Animals were given free access to diets which were freshly prepared and changed every two days.

Blood and tissue sampling. At the end of the feeding period, six rats of each diet group were anaesthetized with an air/diethyl ether mixture, and blood was collected over ethylenediaminetetraacetic acid (EDTA) *via* the abdominal aorta. The samples were immediately centrifuged at

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Abbreviations: AT, adipose tissue; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FA, fatty acids; HPLC, high-performance liquid chromatography; LC, long-chain; NPL, nonphosphorus lipids; PL, phospholipids; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; TL, total lipids; X, xanthine; XO, xanthine oxidase.

TABLE 1

Composition of Dietary Fats[a] and Their Fatty Acid Profiles[b]

Oil	18:2 Diet	18:3 Diet	LCn-3 Diet
Olive	63.7	63.0	63.5
Palm	15.7	17.8	—
Sunflower	20.6	—	8.0
Linseed	—	19.2	—
Menhaden	—	—	28.5
Fatty acid			
14:0	0.2	0.2	1.8
16:0	14.6	15.5	12.3
16:1n-7	0.7	0.6	3.0
18:0	3.9	3.4	3.4
18:1n-9	56.9	56.5	51.9
18:1n-7	2.4	2.6	2.8
18:2n-6	19.7	9.9	10.4
18:3n-3	0.4	10.4	0.8
18:4n-3	—	—	1.1
20:0	0.3	0.3	0.3
20:1n-9	0.2	0.2	0.5
20:4n-3	—	—	0.4
20:5n-3	—	—	4.0
21:5n-3	—	—	0.2
22:5n-3	—	—	0.6
22:6n-3	—	—	4.1
Others	0.7[c]	0.4[c]	2.4[d]
Saturated	19.3	19.6	18.6
n-6 PUFA	19.7	9.9	10.9
n-3 PUFA	0.4	10.4	11.3
n-6 plus n-3 PUFA	20.1	20.3	22.2
PUFA/saturated ratio	1.04	1.04	1.19

[a]Expressed as percentage (w/w) of total dietary fats. PUFA, polyunsaturated fatty acids. LCn-3, long chain n-3 fatty acids.
[b]Expressed as percentage (w/w) of total fatty acids present.
[c]Represented by the sum of the values for 15:0, 16:1n-9, 17:0, 22:0, each being lower than 0.2%.
[d]Represented by the sum of the values for 15:0, 16:1n-9, 16:2n-4, 16:3n-4, 16:4n-1, 17:0, 18:2n-4, 18:3n-6, 18:3n-4, 18:4n-1, 20:1n-7, 20:2n-6, 20:3n-6, 20:4n-6, 20:3n-3, 22:0, 22:1n-11, 22:1n-9, 22:5n-6, 24:0, 24:1n-9, each being lower than 0.2%.

+4°C to collect plasma. After the animals were killed, the hearts were rapidly excised and rinsed in ice-cold saline solution. Liver, brain and adipose tissue were also taken. Plasma and all organs were divided into two portions; one part was frozen in liquid nitrogen and stored at −80°C awaiting vitamin E analysis, and the other part was stored in chloroform/methanol (2:1, vol/vol) prior to lipid analysis. Ten rats from each diet group were sacrificed as described above, but only the hearts were removed and frozen in liquid nitrogen for use in lipid peroxidation studies.

Vitamin E extraction and quantification. Prior to extraction, tissues were homogenized in 3 vol of distilled water. Vitamin E was extracted from the plasma, the heart and the brain, using a method based on that mentioned by Burton *et al.* (12). To 0.5 mL of plasma, or 0.5 mL of tissue homogenate, were added 1 mL of 0.1 M sodium dodecylsulfate (SDS) and 2 mL of ethanol. The mixture was vortex-stirred for 30 s. Then, 1 mL of *n*-heptane was added, the sample was vigorously mixed by vortexing for 30 s and the aqueous and the organic layers were separated by centrifugation for 2 min at 1700 × *g*. The organic layer was carefully drawn off and measured with a 1-mL syringe and transferred to a screw-cap vial. The residue was reextracted two times with 1 mL of *n*-heptane as described above. The extracts were combined and stored at −20°C for analysis.

The method for measuring vitamin E in liver and adipose tissue was adapted from that by Butriss and Diplock (13). To 0.5 mL of tissue homogenate was added 2 mL of 1% pyrogallol in absolute ethanol. After 2 min equilibration at 70°C, 0.3 mL of saturated potassium hydroxide was added, and the resulting mixture was incubated for another 30 min at 70°C. After cooling in ice, 1 mL of distilled water was added, followed by 1 mL of *n*-heptane. The organic layer was removed, and the residue was reextracted two times with 1 mL of *n*-heptane as described above.

Separation and quantification of the four isomers of tocopherol was accomplished by high-performance liquid chromatography (HPLC) coupled with fluorescence spectrophotometric detection (Kontron, Milan, Italy). The analytical column used a 5-μ amino bonded phase (4.6 mm i.d. × 15 cm length). The mobile phase was *n*-heptane/2-propanol (98:2, vol/vol), and the flow rate was 1.6 mL/min. Fluorimetric measurements of tocopherols were done at 285 nm excitation and 325 nm emission. Quantification was done relative to solutions containing known amounts of α, β, γ and δ tocopherol.

Lipid analysis. It has been suggested that expressing vitamin E content as milligrams of tocopherol per gram of fat is a better index than using milligrams per gram of fresh tissue (14). Tissue and plasma total lipids (TL) were therefore extracted according to Folch *et al.* (15), and in the case of tissues total PL were separated from nonphosphorus lipids (NPL) using silicic acid cartridges (16).

In vitro *lipid peroxidation in heart homogenates. In vitro* heart lipid peroxidation was measured on heart homogenates in phosphate buffer (10%, wt/vol). One mL of heart homogenate was incubated in vials in a shaking waterbath at 37°C for 15 or 30 min in the absence (uninduced) or presence (induced) of xanthine/xanthine oxidase/Fe-EDTA (X/XO/Fe-EDTA. 1 mM/0.1 IU per mL/0.2 mM/0.4 mM) as free radical generating system. Heart lipid peroxidation was measured by the thiobarbituric acid reactive substances (TBARS, nmol/g wet weight) assay in the medium after incubation (17).

Statistical analysis. One-way analysis of variance was applied to the data. Group means were compared by the Newman Keuls' test.

RESULTS

Food consumption, animal weight gains and organ weights were similar in the three dietary groups. Only the α-tocopherol isomer could be detected in extracts, and the levels found in plasma and organs were similar or even higher than those found in earlier studies (6,8,18,19). Vitamin E and lipid levels in various organs are shown in Figures 1-4 and Table 2.

Tissues and plasma vitamin E. Heart α-tocopherol content (μg/g wet tissue) was significantly lower when n-3 PUFA were fed to rats (Fig. 1). It decreased by about 20% when the diet was rich in LCn-3. TL, NPL and PL were not significantly affected by the diets (Table 2). The α-tocopherol concentrations expressed as μg/mg of TL (Fig. 2) and NPL (Fig. 3) were not different between the three groups. Only the α-tocopherol concentration expressed

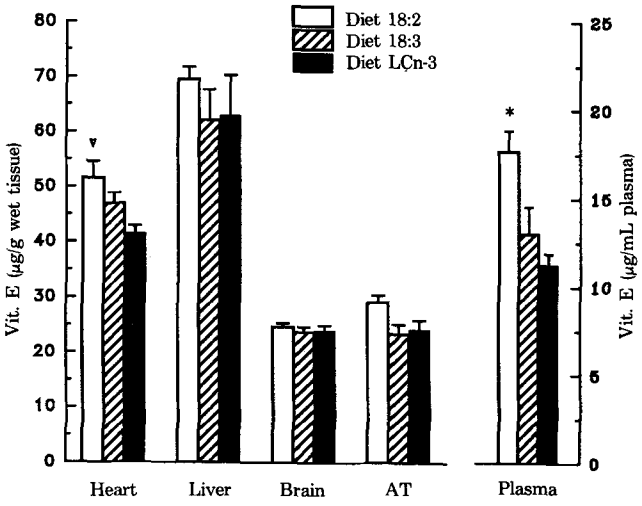

FIG. 1. Total vitamin E (Vit. E) content expressed per g wet tissue or per mL of plasma from rats fed experimental diets. The data are means ± SEM (n = 6). For the plasma, the value bearing an asterisk is significantly different from the other dietary treatments ($P < 0.01$). For the heart, the value bearing a triangle is significantly different from the long-chain n-3 fatty acids (LCn-3 diet) ($P < 0.01$). AT, adipose tissue.

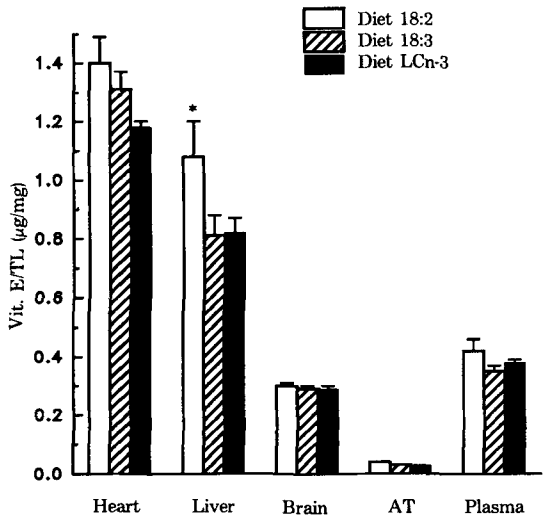

FIG. 2. Total vitamin E content expressed per mg of total lipids (TL) in tissues and plasma of rats fed experimental diets. The data are means ± SEM (n = 6). For the liver, the value bearing an asterisk is significantly different from the other dietary treatments ($P < 0.05$). For abbreviations, see Figure 1.

as μg/mg of PL differed significantly between the 18:2 diet and the LCn-3 diet (Fig. 4).

Liver α-tocopherol concentrations (μg/g wet tissue) were not significantly different in the three diets (Fig. 1). As for the heart, the diets did not significantly modify lipid parameters (Table 2). However, when the liver α-tocopherol concentration was expressed as μg/mg TL, the two n-3 PUFA diets caused significantly lower values (Fig. 2). This decrease could be related to an increase in TL in liver with n-3 FA feeding.

The diets had no effect on α-tocopherol and lipid contents in the brain (Figs. 1-4, Table 2). The α-tocopherol concentration in AT was not affected when n-3 FA were present in the diet (Fig. 1). However, vitamin E content, expressed as μg/mg PL, was significantly decreased by n-3 PUFA diets (Fig. 4). This could be explained by the higher PL content of adipose tissue when n-3 PUFA were added to the diet (Table 2).

Vitamin E concentrations in plasma were significantly decreased by n-3 PUFA (Fig. 1). Lipid levels were also reduced by 30% when the LCn-3 diet was administered (Table 2). Therefore, plasma vitamin E contents when expressed as μg/mg plasma TL were not significantly different from one diet to the other (Fig. 2).

In vitro *heart lipid peroxidation.* As expected, the free radical generating system X/XO/Fe-EDTA enhanced heart lipid peroxidation, but in a time-independent manner. TBARS were significantly higher in the LCn-3 fed animals than in rats fed the 18:2 or 18:3 diets after 15 min at 37°C in either an uninduced or induced system (Fig. 5). The

TABLE 2

Lipid Contents of Heart, Liver, Brain, Adipose Tissue and Plasma After Four Weeks on the Experimental Diets[a]

		18:2 Diet	18:3 Diet	LCn-3 Diet
Heart	TL (mg/g tissue)	37.0 ± 0.6	35.9 ± 0.7	35.1 ± 0.7
	NPL (mg/g tissue)	8.3 ± 0.6	7.7 ± 0.7	7.1 ± 0.4
	PL (mg/g tissue)	28.8 ± 0.5	28.2 ± 0.3	28.1 ± 0.5
Liver	TL (mg/g tissue)	66.6 ± 5.4	76.8 ± 3.8	75.4 ± 5.6
	NPL (mg/g tissue)	34.4 ± 4.8	41.9 ± 4.1	42.1 ± 4.9
	PL (mg/g tissue)	32.2 ± 0.7	34.9 ± 0.7	33.3 ± 1.4
Brain	TL (mg/g tissue)	81.3 ± 0.4	80.6 ± 0.5	81.7 ± 0.6
	NPL (mg/g tissue)	18.1 ± 0.3	18.7 ± 0.3	18.4 ± 0.2
	PL (mg/g tissue)	63.2 ± 0.5	61.8 ± 0.3	63.3 ± 0.5
Adipose tissue	TL (mg/g tissue)	835.0 ± 38.7	891.8 ± 8.6	878.7 ± 9.9
	NPL (mg/g tissue)	826.8 ± 37.6	878.3 ± 8.5	867.3 ± 10.3
	PL (mg/g tissue)	8.2 ± 1.7[b]	13.4 ± 1.3[c]	11.4 ± 0.7[b,c]
Plasma	TL (mg/mL plasma)	4.3 ± 0.4[b]	3.7 ± 0.4[b,c]	2.9 ± 0.1[c]

[a]Values are expressed as means ± SEM (n = 6). Within a line, values bearing different superscript letters differ at $P < 0.05$. Abbreviations: LCn-3, long-chain n-3 fatty acids; TL, total lipids; NPL, nonphosphorous lipids; PL, phospholipids.

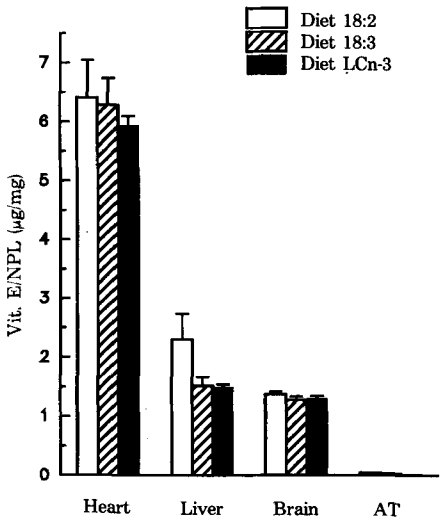

FIG. 3. Total vitamin E content expressed per mg of nonphosphorus lipids (NPL) in tissues of rats fed experimental diets. The data are means ± SEM (n = 6). For other abbreviations, see Figure 1.

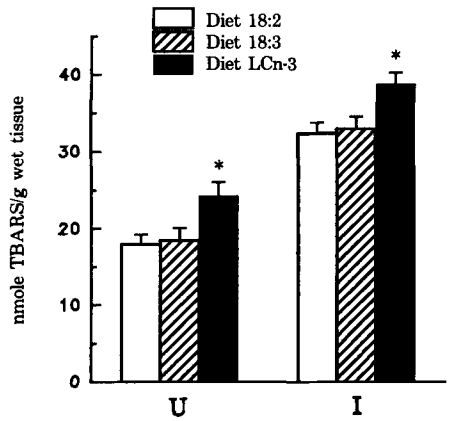

FIG. 5. Lipid peroxidation of heart homogenates from rats fed experimental diets. The data are means ± SEM (n = 10). Heart homogenates were incubated at 37°C for 15 min in the absence (U) or presence (I) of a free radical generating system (1 mM xanthine, 0.1 IU per mL xanthine oxidase, 0.2 mM/0.4 mM Fe/ethylenediaminetetraacetic acid). The values bearing an asterisk are significantly different from the other dietary treatments in the same incubation system ($P < 0.05$). TBARS, thiobarbituric acid reactive substances. LCn-3, long chain n-3 fatty acid.

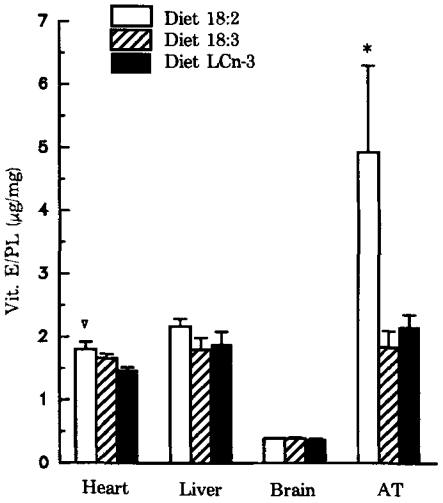

FIG. 4. Total vitamin E content expressed per mg of phospholipids (PL) in tissues of rats fed experimental diets. The data are means ± SEM (n = 6). For the adipose tissue, the value bearing an asterisk is significantly different from the other dietary treatments ($P < 0.01$). For the heart, the value bearing a triangle is significantly different from the LCn-3 diet ($P < 0.05$). For other abbreviations, see Figure 1.

same profiles were observed after 30-min incubation at 37°C, but differences between groups were no longer significant.

DISCUSSION

In the present study we compared the effect of three diets varying in the type and composition of PUFA on vitamin E status and on heart lipid peroxidizability in rats. Membrane PUFA composition and consequently susceptibility to peroxidation is largely dependent on the dietary supply of PUFA. When studying the effect of the type of PUFA administered (*i.e.*, n-6 *vs.* n-3) on both vitamin E status and tissue peroxidizability, care must be taken to feed the rats the same amount of total PUFA. Previous studies (5,6,8,20) have compared diets which supplied total PUFA or saturated FA unequally thus making data interpretation difficult. In the present study, all rats were fed with 20% of the total FA as PUFA as well as saturated FA. Consequently, the PUFA/saturated ratio was the same in the three diets and only the nature of the PUFA varied; 18:2n-6 was partially replaced by 18:3n-3 or LCn-3 PUFA (essentially 20:5 and 22:6n-3). Moreover, our protocol permitted a comparison of n-6 PUFA *vs.* n-3 PUFA on the one hand, and of the precursor (α-linolenic acid) *vs.* its metabolites (essentially EPA and DHA) on the other. In a previous study (3), significant increases in unsaturation in heart PL were seen in rats fed n-3 PUFA in comparison to n-6 PUFA. It was also clearly demonstrated that dietary LCn-3 PUFA had a stronger effect on the fatty acid composition of rat heart PL than their precursor α-linolenic acid which was also present at the same level in the diet. Dietary consumption of LCn-3 PUFA resulted in a much greater incorporation of 20:5n-3 and 22:6n-3 and a much lesser proportion of 20:4n-6 into heart phosphatidylcholine and phosphatidylethanolamine than an equivalent intake of 18:3n-3.

In the present study, it was shown that the type of dietary PUFA also significantly affected the heart vitamin E content which decreased with the n-3 diets, most markedly with the LCn-3 diet. Our data are not in complete agreement with those of Chautan *et al.* (8) who showed that heart α-tocopherol levels in rats fed LCn-3 PUFA were higher than those in rats fed 18:2n-6. In fact, these authors found vitamin E concentrations similar to ours in rats fed LCn-3 diets, but obtained much lower values for animals fed 18:2n-6. This discrepancy between the two sets of data remains to be explained inasmuch as the levels of dietary 18:2n-6 and vitamin E administered to the rats in Chautan's study (8) were higher than

those used in our study. The decrease in heart vitamin E content observed with the n-3 diets in the present study could suggest that more tissue E is required to protect a membrane enriched in n-3 PUFA against peroxidation. Our *in vitro* experiments on heart peroxidation, indeed, confirmed that membranes enriched in n-3 are more susceptible to peroxidation. The data are also in agreement with other studies (5,6) showing that peroxidation in liver and kidney homogenates as well as in liver and heart slices was enhanced by fish oil diets. Disappearance of vitamin E in hearts of rats fed LCn-3 PUFA is therefore likely to be due to mechanisms that take place within the organ, such as peroxyl radical scavenging by vitamin E (21).

Although total plasma vitamin E content was shown to be lower in rats fed LCn-3 PUFA, it was not different from that of the other dietary groups when expressed as μg/mg TL. Our results agree with those by Chantan *et al.* (8) who found the same α-tocopherol/(cholesterol + triglycerides) ratio for all diets they used.

Liver and AT are the major storage sites for α-tocopherol. Both stores tend to be lowered when n-3 PUFA are fed to rats, but the decrease in vitamin E content is significant only if expressed as μg/mg PL in AT or as μg/mg TL in liver. Our results agree with those of other studies which have shown that fish oils lower the α-tocopherol content in the liver of both rats (8) and mice using α-tocopherol supplemented diets (20). However, Leibovitz *et al.* (6) did not find any difference in rat liver α-tocopherol levels following corn oil–lard or menhaden oil diets. In spite of the high levels of vitamin E in the diets (180 mg/kg), the values they found were very low in comparison with ours (about 17 μg vitamin E/g tissue *vs.* more than 60 μg/g), and it cannot be excluded that vitamin E was incompletely extracted. According to Drevan (22), an increased dietary intake of EPA may enhance the demand and utilization of α-tocopherol as an antioxidant by various tissues and, consequently, the amount of vitamin E available for hepatic secretion may be reduced in hepatocytes exposed to dietary EPA.

Brain vitamin E was not affected by dietary PUFA. This could be expected since PUFA composition of brain lipids was not modified by feeding rats the same diets for four weeks (Javouhey-Donzel, A., unpublished data). Many studies have shown that brain fatty acid composition is more resistant to changes in dietary lipids than are other tissues (*e.g.*, Ref. 23).

We conclude that n-3 PUFA diets can influence the vitamin E status of rats even in short-term experiments and that n-3 PUFA can change the susceptibility of the heart to peroxidation. Beneficial cardiovascular effects of fish oils could thus be counterbalanced by an increase in lipid peroxidation. This would suggest that caution would be warranted in using fish oil concentrates as n-3 PUFA supplements of the human diet.

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Dietary Fat Effects on Hepatic Lipid Peroxidation and Enzymes of H_2O_2 Metabolism and NADPH Generation

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The purpose of this study was to determine the effects of dietary fat quantity and fatty acid composition on hepatic H_2O_2-metabolizing systems, activities of NADPH-generating enzymes and lipid peroxidation. One-month-old male C57BL/6J mice were fed one of six diets: (i) 5% fat, rich in 18:2n-6 fatty acid (5% N-6); (ii) 20% fat, rich in 18:3n-3 (N-3); (iii) 20% fat, rich in 18:2n-6 (N-6); (iv) 20% fat, rich in 18:1n-9 (N-9); (v) 20% fat, rich in saturated fatty acids (SAT); and (vi) 20% fat, deficient in essential fatty acids (EFAD); for 11 wk. Comparisons between animal groups receiving different fat quantities showed that activities of glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) and the levels of conjugated dienes were significantly lower in the N-6 than in 5% N-6 group. Conversely, activities of catalase (CAT, EC 1.11.1.6) and selenium-glutathione peroxidase (SeGSHPx, EC 1.11.1.9) were higher in the N-6 than in 5% N-6 group. Among the five dietary groups receiving 20% fat but differing in fatty acid composition, CAT activity was lower in the N-9 group, SeGSHPx activity was lower in the EFAD group, and glutathione reductase (GSSGR, EC 1.6.4.2) activity was higher in the N-6 than in the N-3, N-9, SAT and EFAD groups. The EFAD group had much higher levels of total lipids and conjugated dienes, as well as activities of NADPH-generating enzymes, including G6PDH, ME and isocitrate dehydrogenase (EC 1.1.1.42), than the other four high-fat groups. The hepatic levels of malondialdehyde were not different among the five groups fed 20% fat. In the EFAD group, higher hepatic lipid content can be attributed to higher activities of NADPH-generating enzymes, and the elevation of conjugated diene levels may be related to increased oxygenation of 20:3n-6 (Mead acid) *via* the lipoxygenase/cyclooxygenase pathway. In short, both dietary fat quantity and fatty acid composition selectively affected hepatic H_2O_2-metabolizing systems, activities of NADPH-generating enzymes and lipid peroxidation status.

Lipids 28, 657–662 (1993).

The β-oxidation of fatty acids in peroxisomes is distinct from that of mitochondria in that the location, enzymes involved, end products and regulation of the processes are different between these two systems (1). Of particular interest is that peroxisomal, but not mitochondrial, fatty acyl-CoA oxidase produces H_2O_2 as the by-product (1). Although H_2O_2 is a weak oxidant and is not very active in the aqueous environments of cells, it may cross biological membranes and participate in the iron-catalyzed Fenton reaction, generating highly reactive hydroxyl radicals under physiological conditions (2). Thus, H_2O_2 may cause or promote oxidative damage to macromolecules at sites distal to its production (2).

The primary H_2O_2-detoxifying systems are catalase (CAT) and selenium-glutathione peroxidase (SeGSHPx). CAT is a peroxisomal enzyme and induction of peroxisomal β-oxidation by peroxisome proliferators and high-fat diets is associated with up to a twofold increase in CAT activity (3). SeGSHPx is mainly a cytosolic enzyme capable of reducing H_2O_2 and organic hydroperoxides with the cofactor reduced glutathione (GSH). For the action of SeGSHPx, the intracellular GSH needs to be maintained at a proper concentration, which can be achieved by regenerating from oxidized glutathione (GSSG) *via* NADPH-dependent glutathione reductase (GSSGR) and by synthesis *via* γ-glutamylcysteine synthase (4). The NADPH needed for GSH regeneration may be provided by various NADPH-generating enzymes, such as glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME) and isocitrate dehydrogenase (ICDH) (4). The finding that NADPH is tightly bound to mammalian CAT (5,6) suggests that CAT may also be a NADPH-dependent enzyme. Thus, the provision of NADPH for regeneration of GSH from GSSG, as well as to protect CAT from inactivation by H_2O_2, is of great physiological significance.

Certain high-fat diets, such as diets high in n-3, n-6 or saturated fatty acid (SAT), cause a significant increase in hepatic peroxisomal β-oxidation (7–10) and the activities of CAT and/or SeGSHPx (7,8). High-fat diets have also been shown to affect antioxidant defense mechanisms and lipid peroxidation status (11,12). However, most of these studies used fats varying in several fatty acids (7–12). Thus, a quantitative comparison using one single variable has not been achieved. In the present study we use blends containing comparable fatty acid compositions to study the effects of fat quantity and fatty acid composition on hepatic H_2O_2-metabolizing systems, activities of NADPH-generating enzymes and lipid peroxidation status in C57BL/6J mice.

MATERIALS AND METHODS

Chemicals. Methanol, isobutanol, chloroform, hexane, sulfuric acid and H_2O_2 were purchased from Fisher Scientific (Fair Lawn, NJ). NADPH, NADP, GSSGR, GSH, GSSG, glucose-6-phosphate (G6P), malic acid, isocitrate, 5,5′-dithio*bis*(2-nitrobenzoic acid), bovine serum albumin

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Abbreviations: BSA, bovine serum albumin; CAT, catalase (EC 1.11.1.6); EFA, essential fatty acid; EFAD, essential fatty acid deficiency; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GSH, reduced glutathione; GSSG, oxidized glutathione; GSSGR, glutathione reductase (EC 1.6.4.2); ICDH, isocitrate dehydrogenase (EC 1.1.1.42); MDA, malondialdehyde; ME, malic enzyme (EC 1.1.1.40); SAT, saturated fatty acid(s); SeGSHPx, selenium-glutathione peroxidase (EC 1.11.1.9); TBA, 2-thiobarbituric acid.

(BSA), 1,1,3,3-tetramethoxypropane, trichloroacetic acid and 2-thiobarbituric acid (TBA) were obtained from Sigma Chemical Co. (St. Louis, MO). Ethanol was from Midwest Grain Products of Illinois (Perkin, IL) and potassium dichromate from Allied Chemical Co. (Morristown, NJ). Dietary components were purchased from Teklad Test Diets (Madison, WI). Linseed, safflower, olive, corn and hydrogenated coconut oils were purchased from Bio-Serv (Frenchtown, NJ).

Diet preparation. Modified AIN-76 diets were composed as described in Table 1. High- (20%) and low-fat (5%) diets contained equal amounts of protein, vitamins, minerals and fiber per calorie consumed. The fats used were blends of several plant oils resulting in fats of defined fatty acid compositions. Fat blends contained approximately equal basal amounts of SAT, linoleic acid (18:2n-6), linolenic acid (18:3n-3) and oleic acid (18:1n-9). An essential fatty acid deficient (EFAD) diet was made with hydrogenated coconut oil devoid of unsaturated fatty acids. The basal fatty acids accounted for 11% of the diets, and additional fatty acids (SAT, 18:1n-9, 18:2n-6 or 18:3n-3) accounted for 9% of the total diet. These six diets (detailed in Table 2) were: (i) 5% fat, rich in 18:2n-6 fatty acid (5% N-6); (ii) 20% fat, rich in 18:3n-3 (N-3); (iii) 20% fat, rich in 18:2n-6 (N-6); (iv) 20% fat, rich in 18:1n-9 (N-9); (v) 20% fat, rich in SAT; and (vi) 20% fat, EFAD.

Animal care. Male weaning C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME), five or six per group, were fed one of the six diets (described in Tables 1 and 2) for 11 wk. Feed and water were provided *ad libitum.* In order to minimize autoxidation of dietary lipids, diets were prepared in small batches and stored at -20°C until use. Packets containing one week's worth of food were stored at 4°C, and feed was changed daily. Mice were housed in plastic cages with pine shavings as bedding with a 12-h light/dark cycle.

Tissue preparations and methods. Mice were sacrificed by cervical dislocation following light diethyl ether anesthesia, and the liver was removed immediately. Body and liver weights were recorded. The liver was washed, blotted dry and homogenized in 3 vol of 1.15% KCl in 0.05 M phosphate buffer, pH 7.4. An aliquot of liver homogenate was centrifuged at 9,000 × *g* for 30 min and the supernatant (S9 fraction) stored at −70°C until analyzed. Hepatic GSH (13) and CAT (14) activities were measured in homogenates. Activities of SeGSHPx (15), GSSGR (16), G6PDH (17), ME (18) and ICDH (19) were determined in the S9 fraction. Lipid peroxidation indices were measured by the assays of malondialdehyde (MDA) (20) and conjugated dienes (21) in homogenates. The TBA–MDA complex was detected by high-performance liquid chromatography with fluorescence detection and quantified relative to authentic MDA as standard. Protein was measured by the method of Miller (22) using BSA as standard, and hepatic total lipids by the method of Chiang *et al.* (23) with corn oil as standard.

Statistics. Data are expressed as means ± SD with n = 5–6 per group; differences in mean were analyzed by one-way analysis of variance followed by Newman-Keuls multiple comparison test at $P = 0.05$ (24). Statistical

TABLE 1

Diet Composition

Component	Control (5% fat)	Experimental (20% fat)
High-protein casein	20.0[a]	20.0
Choline bitartrate	0.2	0.2
d,l-Methionine	0.3	0.3
AIN-76A vitamin mix	1.0	1.0
AIN-76 mineral mix	4.0	4.0
Cellulose	4.0	4.0
Corn starch	29.5	16.7
Glucose monohydrate	36.0	20.2
Fat[b]	5.0	16.6
	100.0 g	83.0 g
	3.713 kcal/g	4.468 kcal/g

[a]Amount of each diet component added, in grams.
[b]Specific fat sources for each dietary treatment outlined in Table 2.

TABLE 2

Percent of Diet (w/w) Supplied as Specific Fatty Acids[a]

Dietary group[b]	Basal SAT	Extra SAT	Basal 18:1	Extra 18:1	Basal 18:2	Extra 18:2	Basal 18:3	Extra 18:3
5% N-6	0.6	—	1.4	—	3.0	—	—	—
20% N-3	3.5	—	4.6	—	3.0	—	—	9.0
20% N-6	2.5	—	5.0	—	3.0	9.0	—	—
20% N-9	3.0	—	5.0	9.0	3.0	—	—	—
20% SAT	3.0	9.0	5.0	—	3.0	—	—	—
20% EFAD	3.0	17.0	—	—	—	—	—	—

[a]The fatty acid composition of oil sources was analyzed by gas chromatography on a Shimadzu (Tokyo, Japan) 9A gas chromatograph equipped with a 2-m glass column (3 mm i.d.) packed with Silicon 1Q-Chrom QII 100/120 mesh 275 (Supelco, Bellefonte, PA) and a flame-ionization detector. Temperature was increased from 160 to 210°C at 2°C/min.
[b]5% N-6 group, consisting of four parts safflower oil and one part olive oil. 20% N-3 group, consisting of 16.75 parts linseed oil, 0.25 parts safflower oil and three parts hydrogenated coconut oil. 20% N-6 group, consisting of 12 parts corn oil, 6.5 parts safflower oil and 1.5 parts olive oil. 20% N-9 group, consisting of 14.75 parts olive oil, 0.3 parts safflower oil, 3 parts corn oil and 1.95 parts hydrogenated coconut oil. 20% saturated fatty acids (SAT) group, consisting of 11 parts of hydrogenated coconut oil, 4.5 parts olive oil and 4.5 parts corn oil. 20% essential fatty acid deficient (EFAD) group, consisting of 20 parts hydrogenated coconut oil.

comparisons were performed between the groups 5% N-6 and N-6 and among the five high-fat diet groups N-3, N-6, N-9, SAT and EFAD.

RESULTS

Effects of fat quantity. The effects of dietary fat quantity on various parameters are summarized in Table 3. Mice consuming the N-6 diet had significantly higher body weight than those fed the 5% N-6 diet. However, this change was not reflected in wet liver weight, liver to body weight ratio or hepatic lipid content. The N-6 group had significantly higher activities of CAT (Fig. 1a) and SeGSHPx (Fig. 1b), but significantly lower activities of G6PDH (Fig. 2a) and ME (Fig. 2b), and conjugated diene levels (Table 4) than the 5% N-6 group. No difference was found in GSSGR (Fig. 1c) and ICDH (Fig. 2c) activities, or in GSH (Fig. 1d) and MDA (Table 4) levels.

Effects of fatty acid composition. The effects of enrichment of N-3, N-6, N-9, fatty acids or SAT and deficiency in EFA in the 20% fat diets on body weight, liver weight and hepatic total lipids are summarized in Table 3. The N-3 group had significantly higher body weight than the N-6 and EFAD groups and the N-9 and SAT groups were higher than the EFAD group. The order of body weights is as follows: N-3 N-9 SAT > N-6 > EFAD. However, this effect was not reflected in liver weight or liver to body weight ratios. As for hepatic lipid content, the EFAD group was significantly higher than the N-3, N-6, N-9 and SAT groups, and no difference was found among the latter four groups.

The N-9 group had significantly lower CAT activity than the N-3 and N-6 groups, but not the SAT and EFAD groups; there was no difference in CAT activity among the N-3, N-6, SAT and EFAD groups (Fig. 1a). The EFAD group had a significantly lower activity of SeGSHPx than the other high-fat diet groups (Fig. 1b). The N-6 group had a higher activity of GSSGR than the N-3, N-9 and EFAD groups, but not the SAT group; there was no difference in GSSGR activity among the N-3, N-9, SAT and EFAD groups (Fig. 1c). As for hepatic GSH levels, there was no statistically significant difference among the five groups (Fig. 1d).

Activities of G6PDH and ME were markedly increased in the EFAD group as compared to the N-3, N-6, N-9 and SAT groups (Figs. 2a and b). ICDH activity was also significantly higher in the EFAD group than in N-6, N-9 or SAT, but not in N-3 (Fig. 2c). There were no differences among the latter four groups. The indices of lipid peroxidation are summarized in Table 4. Conjugated diene levels were significantly higher in the EFAD group than in the N-3, N-6, N-9 and SAT groups, and there was no difference in the levels of MDA among all five groups.

DISCUSSION

To better understand the effects of dietary fat quantity and fatty acid composition on hepatic H_2O_2 metabolism, we have devised several diets containing fat blends differing in only one type of fatty acid. Our results show that even though high-fat diets increase mouse body weight, not all high-fat diets of the same quantity affect body weight to the same extent. For instance, the high-fat diet rich in linolenic acid was most effective in increasing body weight, while the high-fat diet rich in linoleic acid or EFAD was less effective.

Similarly, both fat content and fatty acid composition can affect the activities of H_2O_2-metabolizing enzymes in a selective manner. For example, where other studies showed that the activities of CAT and SeGSHPx were increased in animals fed corn oil or other fats high in polyunsaturated fatty acids (7–10), our results indicate that not all high-fat diets increase these two enzyme activities. In our study, a high-fat diet rich in linoleic or linolenic acid increased activities of SeGSHPx and CAT. The latter activity is frequently used as an indicator of peroxisome induction (3). Our results, however, also suggest that a high-fat diet rich in oleic acid does not induce CAT activity and perhaps peroxisomal β-oxidation to the same extent as other diets rich in either linoleic acid or linolenic acid. Thus, oleic acid is probably not a good substrate for peroxisomal β-oxidation *in vivo*. Indeed, this is supported by *in vitro* evidence showing that linoleic and linolenic acids were β-oxidized more quickly than oleic acid by peroxisomal β-oxidation enzymes (25,26). Additionally, the EFAD group had lower SeGSHPx activity than the other

TABLE 3

Effects of Dietary Fat Quantity and Fatty Acid Composition on C57BL/6J Mouse Body Weight (g), Liver Weight (g and %) and Hepatic Lipid Content (mg/g)

Dietary group	Body weight[a] (g)	Liver weight (g)	Liver weight (%)[b]	Hepatic lipids (mg/g)
5% N-6	26.9 ± 2.3	1.49 ± 0.21	5.47 ± 0.95	104 ± 11
20% N-3	35.9 ± 2.0[d]	1.59 ± 0.18[d]	4.53 ± 0.36[d]	75 ± 6[d]
20% N-6	31.2 ± 2.4*[e,f]	1.57 ± 0.23[d]	5.04 ± 0.62[d]	80 ± 11[d]
20% N-9	34.5 ± 2.5[d,e]	1.70 ± 0.23[d]	4.92 ± 0.31[d]	90 ± 15[d]
20% SAT[c]	34.0 ± 4.8[d,e]	1.74 ± 0.35[d]	5.15 ± 0.91[d]	96 ± 19[d]
20% EFAD[c]	29.0 ± 2.2[f]	1.65 ± 0.16[d]	5.69 ± 0.36[d]	146 ± 23[e]

[a]Data represent the means and SD of five or six mice maintained on the respective diets for 11 wk. Statistical analysis was performed by analysis of variance coupled with Newman-Keuls multiple comparisons test at $P = 0.05$ (Ref. 24). Significant differences between the 5% N-6 and 20% N-6 groups are indicated by an asterisk; differences among all 20% fat diet groups are indicated by different superscript letters (d–f).
[b]Expressed as percent of body weight.
[c]Abbreviations as in Table 2.

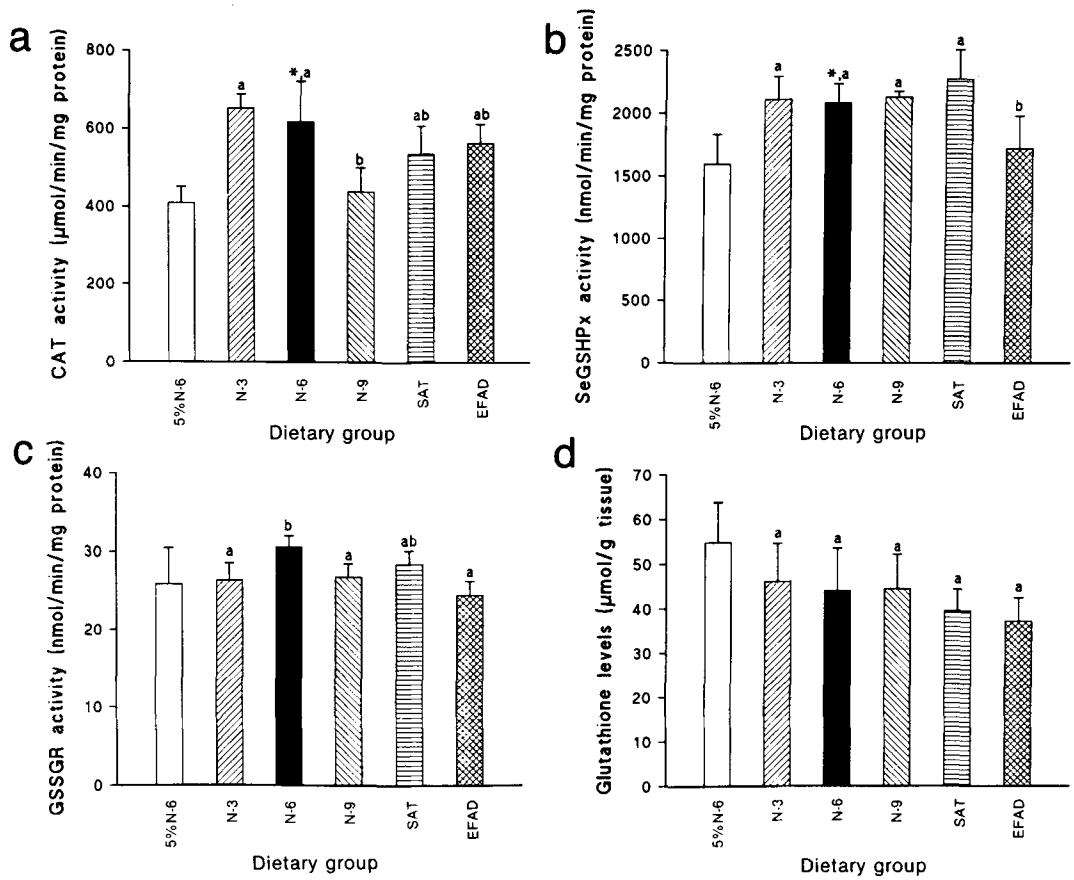

FIG. 1. Effects of dietary fat quantity and fatty acid composition on hepatic (a) catalase (CAT), (b) selenium-glutathione peroxidase (SeGSHPx), (c) glutathione reductase (GSSGR) activities and (d) on reduced glutathione levels. For 11 wk, male weaning C57BL/6J mice were fed one of the six diets described in Tables 1 and 2. Other treatments are detailed in the Materials and Methods section. Each column and bar represent the mean and SD of five or six animals. Differences in means were analyzed by one-way analysis of variance coupled with Newman-Keuls multiple comparisons test at $P = 0.05$ (Ref. 24). An asterisk indicates significant difference between the means of the 5% N-6 and the N-6 groups. The differences among the five 20% fat diet groups are denoted by different letters. Throughout all the figures: 5% N-6 signifies a 5% fat diet rich in N-6 fatty acid; N-3 signifies 20% fat rich in N-3 fatty acid; N-6 signifies 20% fat rich in N-6 fatty acid; N-9 signifies 20% fat rich in N-9 fatty acid; SAT signifies 20% fat rich in saturated fatty acid; and EFAD signifies 20% fat deficient in essential fatty acids.

TABLE 4

Hepatic Lipid Peroxidation Indices in C57BL/6J Mice Fed Diets Containing Different Fat Quantities and Fatty Acid Compositions

Dietary group	Malondialdehyde[a] (nmol/g tissue)	Conjugated dienes[b] (unit/mg lipid)
5% N-6	16.8 ± 5.0	0.425 ± 0.008
20% N-3	17.6 ± 3.6[d]	0.311 ± 0.003[d]
20% N-6	15.7 ± 2.2[d]	0.298 ± 0.001[d]
20% N-9	15.9 ± 2.2[d]	0.344 ± 0.010[d]
20% SAT[c]	17.6 ± 3.3[d]	0.334 ± 0.002[d]
20% EFAD[c]	15.2 ± 2.7[d]	0.543 ± 0.014[e]

[a]Data represent the means and SD of five or six mice maintained on the respective diets for 11 wk. Data were analyzed by analysis of variance coupled with Newman-Keuls multiple comparisons test (Ref. 24). Significant differences ($P < 0.05$) between the means of the 5% N-6 and 20% N-6 groups are indicated by an asterisk and the comparison among all 20% fat diet groups by different superscript letters (d–e).

[b]Expressed as the ultraviolet absorbance at 234 nm per mg lipid.

[c]Abbreviations as in Table 2.

four high-fat diet groups, indicating that EFA are necessary for the increase in SeGSHPx activity in mice fed high-fat diets. Fat content of the diet did not affect GSSGR activity except that enrichment with linoleic acid (n-6 fatty acid) increased it. The reason why enrichment with linoleic acid increased GSSGR activity is not clear at present.

The pentose phosphate pathway is involved in several functions, including regenerating GSH for the degradation of H_2O_2 by SeGHSPx (4). However, the provision of NADPH for the metabolism of hydroperoxides may not be solely dependent on the pentose phosphate shunt, as in the case of red blood cells (4). Other enzymes, such as ME and ICDH, may provide some NADPH for GSH regeneration (4,27). Induction of peroxisomes by peroxisome proliferators often accompanies the induction of ME and G6PDH (28–31). Dietary fat content is known to affect hepatic lipogenesis. In particular, high-fat diets suppress activities of lipogenic enzymes, *i.e.*, G6PDH and ME (32). Although high-fat diets and peroxisome proliferators

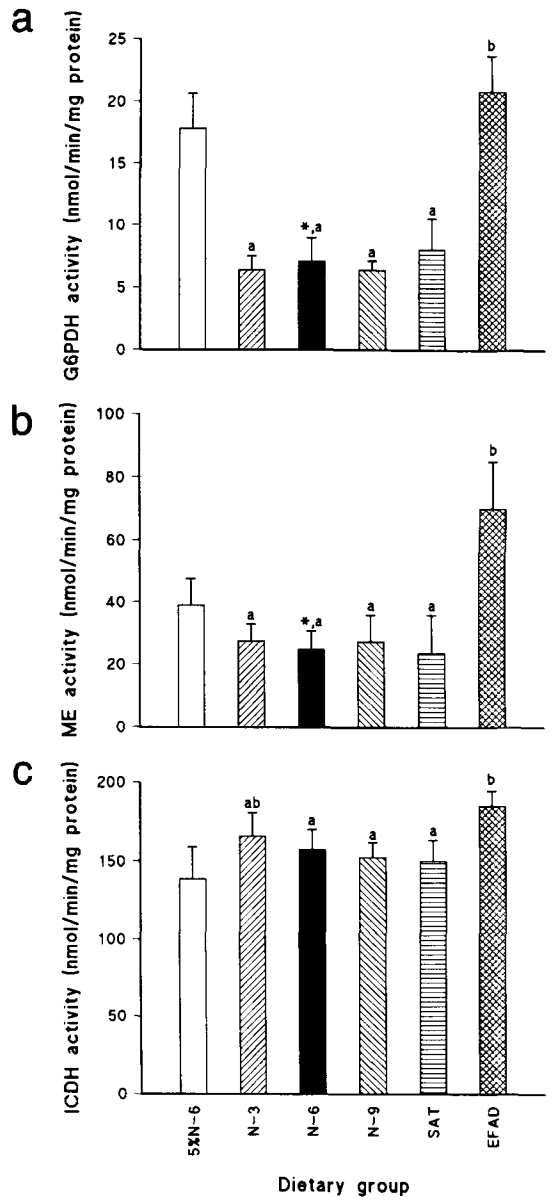

FIG. 2. Effects of dietary fat quantity and fatty acid composition on hepatic (a) glucose-6-phosphate dehydrogenase (G6PDH), (b) malic enzyme (ME) and (c) isocitrate dehydrogenase (ICDH) activities. For details of diet preparation and animal treatment see the Materials and Methods section. Data are the means and SD of five or six mice. Significant differences in means are indicated by an asterisk or different letters as analyzed by one-way analysis of variance and Newman-Keuls multiple comparisons test at $P = 0.05$ (Ref. 24). All symbols and designations are the same as described for Figure 1.

similarly induce peroxisomal β-oxidation, they differ in their effects on ME and G6PDH activities. Nevertheless, EFA deficiency overcomes this suppression and increases the activities of all three NADPH-generating enzymes. This may be due to the increased lipogenesis and synthesis of 20:3n-6 (Mead acid) (33). It has been shown that ICDH and enzymes of the pentose phosphate pathway are present in peroxisomes (34). Moreover, treatment with the peroxisome proliferator clofibrate increases peroxisomal, but not cytosolic, G6PDH activity (34). It is possible that increased H_2O_2 production in peroxisomes may subsequently increase the oxidation of NADPH and activate antioxidant defense mechanisms, such as the pentose phosphate shunt, GSH regenerating pathways and SeGSHPx.

It is interesting that mice fed the 5% N-6 diet or the EFAD diet had significantly higher levels of hepatic conjugated dienes. A similar effect was also seen with hepatic lipid content in these two groups. Although NADPH-producing capacity was elevated in the EFAD and 5% N-5 groups, it did not help prevent conjugated diene formation. However, the greatly increased levels of conjugated dienes in the EFAD groups may not be due to lipid peroxidation and/or impairment of antioxidant defense mechanisms. First, the hepatic vitamin E levels in the EFAD groups were approximately twice as much as the other four high-fat diet groups (data not shown), without impairment of other antioxidant defense mechanisms. Secondly, the source of dienes are unlikely of dietary fat origin, as in the case of rats fed choline devoid diet (35). We hypothesize that the accumulation of conjugated dienes is due to increased oxygenation of 20:3n-9 fatty acid *via* the cyclooxygenase and/or lipoxygenases. This particular fatty acid is not normally seen in animal tissues except when devoid of EFA (33). There is evidence showing that 20:3n-9 can be metabolized to a variety of hydroxy eicosanoids by cyclooxygenase (36) and leukotriene A_3 by 5-lipoxygenase (37). However, it is known that the measurement of conjugated dienes is subject to interference of various origin (38). Further chemical analyses are needed to confirm this effect. Whether this mechanism of enzymatic oxygenation (to linoleic acid and its elongated and desaturated derivatives) can be attributed to the increase in conjugated diene levels in the 5% N-6 group also warrants exploration.

Both fat quantity and fatty acid composition were expected to affect the generation of lipid peroxidation products. However, dietary fat was not found to cause any difference in MDA levels. Generally, it is assumed that the formation of conjugated dienes will eventually lead to β-scission of lipid hydroperoxides leaving at least two double bonds, forming MDA (39). Similar to the cases of EFAD and the 5% N-6 groups in this study, treatments with peroxisome proliferators have been shown to elevate hepatic levels of conjugated dienes without increasing MDA levels (40–42). Elevated conjugated diene levels were not reflected in increased MDA levels in our study or in previous investigations. Perhaps the liver possesses active enzymatic systems for aldehydic products (43), which is inducible by treatment with peroxisome proliferators (44). Also, the procedure we used for the measurement of MDA was more specific (20), which may partly explain our findings.

In summary, the levels of dietary fat and fatty acid compositions were found to selectively affect hepatic H_2O_2-metabolizing systems, activities of NADPH-generating enzymes and lipid peroxidation status. The 5% N-6 and EFAD groups had higher activities of NADPH-generating enzymes than their respective counterparts; consequentially, both groups also had higher hepatic lipid content. EFAD resulted in elevated levels of conjugated dienes, possibly due to increased oxygenation of 20:3n-9 *via* the lipoxygenase/cyclooxygenase pathway.

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Diabetes Increases Excretion of Urinary Malonaldehyde Conjugates in Rats[1]

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The effect of streptozotocin-induced diabetes on the urinary excretion of thiobarbituric acid test-positive materials was examined. In diabetic rats, urinary excretion of thiobarbituric acid reactive substances was increased 5-fold over that in nondiabetic animals. High-performance liquid chromatography of urine samples revealed that five of the six fractions previously found to be increased in vitamin E deficiency [Lee, H.-S., Shoeman, D.W., and Csallany, A.S. (1992) *Lipids 27,* 124-128] were also significantly increased in streptozotocin-induced diabetes. The data suggest that a high level of oxidative stress is induced by uncontrolled diabetes in rats.
***Lipids 28,* 663-666 (1993).**

Lipid peroxidation, the nonenzymatic autooxidation of polyunsaturated fatty acids, has numerous deleterious effects on biological systems (1) and has been implicated in the process of aging as well as the pathogenesis of several diseases (2). One of the most studied products of lipid peroxidation is malonaldehyde (MDA), a dialdehyde known to react with proteins (3-5) and amino acids (6,7). Several studies have shown a positive relationship between *in vivo* lipid peroxidation and urinary excretion of MDA (8,9).

Diabetes mellitus is a disease characterized by a state of prolonged hyperglycemia. Jain (10) has demonstrated that red blood cells exposed to high concentrations of glucose (45 mM) *in vitro* showed increased levels of membrane peroxidation. Further, the level of thiobarbituric acid reactive substances (TBARS), which include MDA, was found to be higher in the red blood cells of streptozotocin-induced diabetic rats compared to nondiabetic animals (11). Serum TBARS have also been reported to be increased in diabetes, both in rats (12) and humans (13). These results suggest that diabetes increases oxidative stress.

Recently, Lee *et al.* (14) have fractionated the TBARS from urine of normal and vitamin E-deficient rats using high-performance liquid chromatography (HPLC). They demonstrated that, in the state of increased oxidative stress caused by vitamin E deficiency, the amount of several of these materials was significantly increased. The purpose of the present study was to determine if a similar increase occurs under another condition of oxidative stress, *i.e.*, in streptozotocin-induced diabetes.

METHODS AND MATERIALS

Animals. Male Wistar rats (initial weight, 150-175 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed individually in suspended wire-mesh stainless steel cages in a temperature-controlled room (22°C) with a 12-h light/dark cycle. Animals were given *ad libitum* access to food and water.

Experimental design. All animals were fed the semi-purified diet prior to the beginning of the experiments to allow adaptation to the diet and environment. The diet consisted of (g/kg): cornstarch, 650; casein, 200; cellulose, 50; corn oil, 50; AIN-76 mineral mix (U.S. Biochemical Corp., Cleveland, OH), 35; AIN-76 vitamin mix (U.S. Biochemical Corp.), 10; DL-methionine, 3; and choline bitartrate, 2. This diet provides 50 mg rac-α-tocopherol acetate per kg, equivalent to 35 mg *D*-α-tocopherol per kg, from the vitamin mix and approximately 9 mg per kg diet of α-tocopherol activity from the corn oil, for a total α-tocopherol equivalent of 44 mg/kg diet. Butylated hydroxytoluene (10 mg/kg) and menadione sodium bisulfite (1.33 mg/kg) were also added.

In the first experiment, the animals were adapted to the semi-purified diet for 9 d before administration of intraperitoneal injections of either streptozotocin (60 mg/kg body wt in 0.1 M citrate, pH 4.5) to induce diabetes, or a sham injection of citrate buffer alone. All animals continued on the semi-purified diet. After 12 wk, the animals were anesthetized, blood was collected by cardiac puncture for determination of glycated hemoglobin (GHb) and both kidneys were removed and weighed.

Body weights were determined at the beginning of the experiment, at week 2 and weekly thereafter. Twenty-four hour food intake was determined once a week at week 2 and weekly thereafter. At the beginning of the experiment and weekly thereafter, the animals were transferred to stainless steel metabolic cages once a week. Twenty-four-hour urine samples were collected and the volumes accurately measured. The collection from week 11 was centrigued to remove debris and an aliquot was frozen at −20°C for determination of the urinary excretion of TBARS and for fractionation by HPLC.

A second experiment was conducted to determine whether diuresis *per se* was responsible for the increase in urinary TBARS found in the diabetic animals. Male Wistar rats were adapted to the semi-purified diet for 10 d. The animals were housed in metabolic cages and given intraperitoneal injections of hydrochlorothiazide (120 mg/kg dissolved in PEG 200) (Sigma Chemical Co., St. Louis, MO) four times over 24 h. Control animals were injected with vehicle only. Body weight at the time of injection ranged from 220-270 g. Twenty-four hour urine samples were collected, the volume noted and aliquots stored at −20°C until analyzed for urinary TBARS.

Analyses. GHb was determined on whole blood by affinity chromatography using a commercial kit (GlycAffin, Isolabs, Akron, OH).

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Abbreviations: GHb, glycated hemoglobin; HPLC, high-performance liquid chromatography; MDA, malonaldehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

The levels of TBARS in urine samples were measured by the modified filtration procedure of Tarladgis *et al.* (15), as described by Lee *et al.* (14). Chromatographic fractionation and free MDA analysis of urine samples were carried out by HPLC using a TSK G1000 PW column (7.5 mm i.d. × 30 cm) (Beckman Instruments, Berkeley, CA) with a mobile phase of 0.1 M Na_3PO_4 buffer, pH 8.0, at a flow rate of 0.6 mL/min, and monitoring the eluant at 267 nm (16).

Statistics. Group means were compared by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

As shown in Figure 1, the diabetic animals exhibited typical signs of diabetes. Panel A shows that the control group gained weight steadily over the course of the experiment, whereas the diabetic group gained relatively little weight. Food intake (Panel B) was greatly increased in the diabetic group compared to the control group during the entire 12-wk experiment. Twenty-four hour urine volume increased immediately in the diabetic compared to the control group and remained elevated throughout the 12-wk experiment (Panel C). The urine volume in the diabetic group compared to that in the control group was significantly greater at each week except week 8.

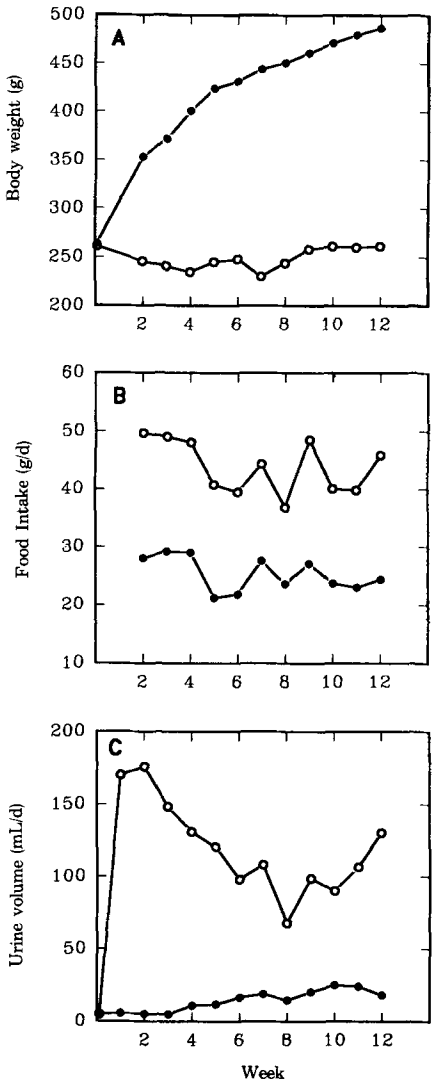

FIG. 1. Body weight change (Panel A), food intake (Panel B) and 24-h urine volume (Panel C) in normal (●) or diabetic (○) rats. Values are means of eight to nine animals per group. Body weight and food intake differences between the control group and the diabetic group were statistically significant ($P < 0.05$) at all time points, as judged by Student's *t*-test. Urine volume differences between the groups were statistically significant ($P < 0.05$) at all time points except week 8.

The GHb values and kidney weights (per 100 g body weight) are shown in Table 1. GHb is a measure of long-term blood glucose level used routinely in monitoring human diabetics and reflects the time-averaged blood glucose concentration over the preceding 60 to 90 d (17). The degree of glycation correlates well with other measures of diabetic control, such as fasting blood glucose concentration, response to a glucose tolerance test and urinary glucose excretion (18,19). The mean hemoglobin glycation of ≈14% in the diabetic group indicates that these animals had experienced high plasma glucose concentrations. The diabetic group also showed an increase in relative kidney weights compared to the control group. Renal enlargement in rats is known to begin within days of the induction of diabetes and continues for at least 6 wk (20). The degree of enlargement has been correlated to the degree of glycemic control, as measured by fasting plasma glucose concentration (21). The significantly enlarged kidneys in the diabetic animals relative to the nondiabetic controls further confirm the diabetic state of these animals.

Diabetic rats excreted approximately five times more urinary TBARS per 24 h compared to nondiabetic controls, a statistically significant increase (Fig. 2). Draper *et al.* (4) had demonstrated that most (75%) of the MDA-containing components of rat urine was present as *N*-α-acetyl-ε-(2-propenal)lysine. They further demonstrated that this MDA adduct increased with the feeding of a highly polyunsaturated fat. None of the nondiabetic animals and only two of six diabetic animals had detectable amounts of free MDA in urine when analyzed by HPLC. The absence of free MDA could result from the fact that most MDA is rapidly metabolized by the liver (22) and thus only a very minor fraction of the body burden of MDA is excreted in the urine. Another possibility is that the reactivity of free MDA might lead to its rapid combination with other compounds and excretion in the urine in a derivatized form (23). This MDA would be released from its derivatized forms by acid hydrolysis during the determination of TBARS. A third possibility

TABLE 1

Glycated Hemoglobin and Kidney Weight in Normal and Diabetic Rats After 12 Weeks[a]

	Normal	Diabetic
Glycated hemoglobin (%)	3.9 ± 0.1	14.4 ± 0.5[b]
Kidney weight, g/100 g body weight	0.67 ± 0.06	1.33 ± 0.03[b]

[a]Values are means ± SEM of eight to nine animals per group.
[b]Significantly ($P < 0.001$) different from the normal group by Student's *t*-test.

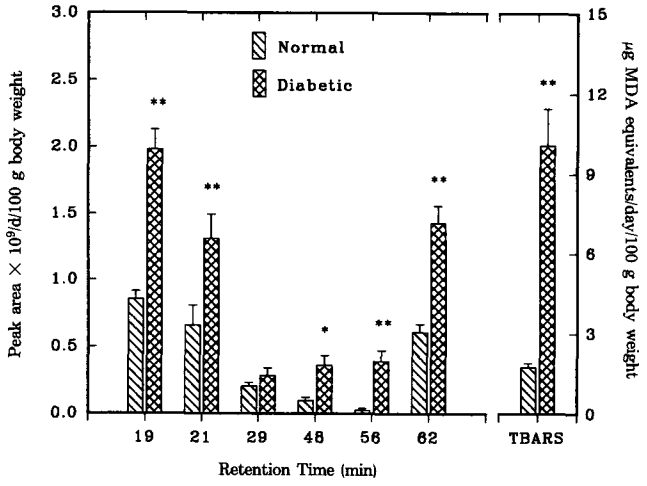

FIG. 2. Comparisons of high-performance liquid chromatography (HPLC) separated urinary carbonyl-containing compounds, as measured by absorbance at 267 nm, and of urinary excretion of thiobarbituric acid reactive substances (TBARS), both per 24 h per 100 g body weight in normal and diabetic rats. Values represent means ± SEM of five to six animals per group for HPLC fractions and eight to nine animals per group for TBARS. *$P < 0.002$; **$P < 0.001$, by Student's *t*-test. MDA, malonaldehyde.

is the presence in urine of certain carbonyl compounds besides derivatized MDA (14). The carbonyl groups present in urine could be derived from short- or medium-chain secondary lipid peroxidation products which also react with the TBA reagent.

To examine the possibility that the increased urinary TBARS in the diabetic animals were due only to diuresis, diuresis was induced in normal animals with hydrochlorothiazide. Table 2 shows that in these nondiabetic animals, the increase in urine volume of >250% was not accompanied by a statistically significant ($P = 0.22$) increase in urinary TBARS. Thus, diuresis *per se* does not appear to influence urinary TBARS.

In a previous study, urine was fractionated on a Spherogel-TSK 1000 PW column and the effluent monitored at 267 nm. In this system, free MDA (when present) is well separated [retention time (R_t), 44 min] from a number of other fractions found in urine (14). The areas of six of these fractions (R_t, 19, 21, 29, 48, 56, 62 min) were larger in the vitamin E-deficient group than in the control group. It was suggested that these six urine fractions are vitamin E-deficiency dependent, and therefore may be related to *in vivo* lipid peroxidation.

TABLE 2

Urine Volume and 24-Hour Urinary TBARS in Normal and Hydrochlorothiazide-Treated Rats[a]

	Control	Hydrochlorothiazide treated
Urine volume (mL/24 h)	17.7 ± 2.0	46.6 ± 4.9[b]
Urinary TBARS (Mg equiv. MDA/24 h/100 g body weight)	3.5 ± 0.6	4.5 ± 0.5

[a]Values are means ± SEM of five animals per group.

[b]Significantly ($P < 0.001$) different from the control group by Student's *t*-test.

In the present study, the areas of five of six peaks previously identified as lipid peroxidation-related were significantly increase ($P < 0.002$) (Fig. 2) in diabetic rats over nondiabetic controls. The magnitude of the difference between the groups in this study was equal to or greater than the difference between the normal and vitamin E-deficient animals found in the previous study. This suggests that diabetes induced by streptozotocin increase *in vivo* lipid perioxidation to a level comparable to that which occurs during severe vitamin E deficiency.

The increase in *in vivo* peroxidation in diabetes could be due to elevated blood glucose concentrations. Jain *et al.* (11) found increased red blood cell thiobarbituric acid (TBA) reactivity *in vitro* with increasing concentrations of glucose. TBA reactivity of red blood cells was also increased in streptozotocin-induced diabetic rats compared to non-diabetic animals (12). However, in animals whose blood glucose concentration was normalized by insulin treatment, no significant increase in TBA reactivity occurred (12).

How elevated blood glucose concentrations might increase the oxidative stress of an animal remains to be established. However, Hunt *et al.* (24) have presented evidence from *in vitro* studies that glucose undergoes metal-catalyzed autooxidation reactions leading to the production of hydroxyl radicals. These highly reactive radicals are known to attack polyunsaturated fatty acids in biomembranes, inducing increased lipid peroxidation. In this way, high blood glucose concentrations could produce a metabolic state similar to vitamin E deficiency. Whether increased levels of chelating agents or quenchers of free radicals such as vitamin E could interrupt this sequence of events, and thereby reduce the oxidative stress of diabetes, is a question deserving further study.

ACKNOWLEDGMENTS

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METHOD

A Multivariate Optimization of Triacylglycerol Analysis by High-Performance Liquid Chromatography

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In the present study we have used statistical experimental design and multivariate optimization to formally optimize a reversed-phase high-performance liquid chromatography method for the analysis of triacylglycerol molecular species of natural oils. The optimal conditions found were, on an octadecylsilan-column, from acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol), at a column temperature of 50°C and a flowrate of 1.5 mL/min using a negative exponential gradient profile. Several examples of separations of natural seed and animal oils, *i.e.*, soybean oil, rapeseed oil, palm oil, linseed oil, tallow and fish oil, are given. A version of the equivalent carbon number concept, utilizing the Snyder polarity index, was used to identify the molecular species. *Lipids 28,* **667–675 (1993).**

Triacylglycerols (TAG) are the most abundant lipid class found in nature. TAG are also the most complex with n^3 potential molecular species possible, n being the number of fatty acids. The TAG molecular species profile represents a key to the understanding of the physical characteristics of an oil and also is a unique means of identification which has been utilized in various instances (1,2). The degree of certainty in the identification is dependent upon the complexity of the oil and on the degree of separation attainable in a given chromatographic system. In order to minimize ambiguity, optimal separation between molecular species is desirable. This has been accomplished in some cases by high resolution gas chromatography (HRGC) at temperatures above 300°C (3). However, due to the tendency of species containing polyunsaturated fatty acids to decompose at high temperatures, there has been a major trend toward the use of reversed-phase high-performance liquid chromatography (RP-HPLC) (2,4–9).

Choosing the solvent system in HPLC is often a time-consuming and tedious chore which requires the localization of the region of optimal separation in the multidimensional solvent mixture space. Statistical experimental design and multivariate modeling (10–15) have been shown to drastically reduce the experimental task to arrive at optimal conditions.

The choice of the solvent system is also dependent on the mode of detection used. A lack of strong chromophores in TAG molecules makes ultraviolet (UV)-detection difficult. In addition, in UV-dectection the response depends on the degree of unsaturation in a given molecular species, which can be highly variable. Refractive index detection limits the choice of solvent to isocratic systems resulting in a concomitant loss in resolution. The light-scattering detector has gained in popularity due to its ability to permit gradient elution and thus comes close to the ideal detector for lipid analysis. It is not without drawbacks, however, as its response function is sigmoidal in shape, necessitating careful calibration for quantitative work.

In RP-HPLC of TAG molecular species, retention is a function of both the total chainlength of the fatty acyl moieties and the total number of double bonds. Increasing chainlength increases the elution time while increasing the degree of unsaturation reduces the elution time. This relationship has been utilized in the partition number (PN) concept (16), a general rule of thumb being that $PN = CN - 2 \times U$, where CN is the total number of carbons and U is the total number of double bonds in the fatty acyl chains. This simple relationship can be extended into the equivalent carbon number (ECN) concept, where differences between species of equal PN are taken into consideration. This last relation is empirically derived by building a mathematical model between the retention times of a set of saturated standards and their respective ECN values (1,2,5,9,17,18), thereby enabling one to predict ECN values for unknown species from their retention times.

The goal of the present study was to optimize an existing RP-HPLC system developed in our laboratory for the analysis of natural TAG oils utilizing statistical experimental design and response surface modeling. The utility of the method is illustrated for the analysis of several natural TAG oils, *i.e.*, soybean oil, rapeseed oil, palm oil, linseed oil, tallow and fish oil. The separated peaks were identified by their ECN values.

MATERIALS AND METHODS

Materials. Chromatographically purified natural oils were obtained from Karlshamns LipidTeknik AB (Stockholm, Sweden). TAG standard mixtures were obtained from Larodan (Malmö, Sweden) and from Sigma (St. Louis, MO). Acetonitrile and isooctane were of analytical grade and purchased from E. Merck (Darmstadt, Germany). Ethanol was of spectroscopic grade (99.5 vol%) from Kemetyl (Stockholm, Sweden). A mixture of standards containing the saturated molecular species tridecanoin (DDD), trilaurin (LaLaLa), trimyristin (MMM), tripentadecanoin (PdPdPd), tripalmitin (PPP), triheptadecanoin (HdHdHd) and tristearin (StStSt) was used to develop the ECN models.

HPLC. The HPLC system consisted of two Shimadzu LC-6A pumps (Kyoto, Japan) integrated with a Shimadzu SCL-6B system controller and an ODS-column (Superspher RP-100, 250 × 4.6 mm, 100 Å) from Merck. The column was thermostatted with a water bath. Detection was

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Abbreviations: Ad, arachidic acid; CN, carbon number; CRS, chromatographic resolution statistic; D, decanoic acid; ECN, equivalent carbon number; Hd, heptadecanoic acid; HPLC, high-performance liquid chromatography; HRGC, high resolution gas chromatography; L, linoleic acid; La, lauric acid; Ln, linolenic acid; M, myristic acid; Nd, nonadecanoic acid; O, oleic acid; P, palmitic acid; P', Snyders polarity index; Pd, pentadecanoic acid; PN, partition number; PLS, partial least squares correlations in latent variables; P, palmitic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; St, stearic acid; TAG, triacylglycerol; UV, ultraviolet.

TABLE 1

Reduced Factorial Designs of the First[a] Experimental Block

Run number	EtOH (vol%)	Isooctane (vol%)	Temperature (°C)	Flowrate (mL/min)	Gradient[b] profile	B% endpoint	CRS[c] value
1	45	15	50	1.5	0	100	8.935
2	45	25	50	0.8	−4	50	10.556
3	45	25	70	0.8	0	100	—
4	35	25	70	1.5	0	50	—
5	45	15	70	1.5	−4	50	—
6	35	25	50	1.5	−4	100	7.634
7	35	15	70	0.8	−4	100	—
8	35	15	50	0.8	0	50	10.640

[a]Plackett-Burman.
[b]Gradient: A to B in 80 min.
[c]CRS, chromatographic resolution statistic.

done with a light-scattering detector from Cunow, model DDL 11 (Cergy, St. Christophe, France). The detector nebulizer was set at 35 psi and held at a temperature of 90°C. Collection and integration of data was done on the PC integration Pack (Kontron, Milan, Italy).

Sample preparation. Twenty-five mg of the oil was dissolved in 5 mL of ethanol/isooctane (3:1, vol/vol). One hundred mg of each standard mixture was dissolved in 25 mL ethanol/ isooctane (3:1, vol/vol), and 10 μL of each sample was injected.

Multivariate methods. These methods have been described in detail in the literature (19–21), and their use in lipid analysis has recently been reviewed (22). In brief, the underlying strategy of partial least squares correlations in latent variables (PLS), which is a widely used method for multivariate calibration, is to consider each experimental variable (vol% of a solvent, etc.) and response variable (peak resolution, etc.) as coordinate axes in two separate multidimensional spaces. The goal is thereafter to build the defining relationship between the two different blocks of variables, *i.e.,* the independent and the dependent blocks. Each object (object = sample) is described by a vector containing the values of the experimental variables or the response variables and can thus be seen as a point in each respective space. A set of objects with the same measured variables form a swarm of points in this space and objects similar to each other will be near to each other, so that distance therefore constitutes a measure of similarity/dissimilarity. New variables representing a maximum amount of the systematic variance present in the data are calculated as linear combinations of the experimental and response variables. These are often called the latent factors, which describe the intrinsic, underlying variance structure of the system under study. The defining relationship between the two blocks is built by using information from the dependent block to adjust or rotate the latent factors from the independent block until maximal linear correlation is obtained between the blocks. PLS thus models both the dependent and independent blocks simultaneously. The PLS method can also be used to extract certain specified portions of the systematic variance from the experimental data (19–22).

Optimization procedure. Optimization of an existing method was done in two sequential steps utilizing statistical experimental design, response-surface modelling (10,11,22) and PLS regression (19,20). Optimization was done using chromatographically purified soybean oil throughout the study. Calculations were done in Excel (Microsoft, Seattle, WA); response surface modelling was done in Statgraphics (STSC, Rockville, MD); PLS regression was done in SIMCA 3B (Sepanova, Enskede, Sweden) and calculation of ECN values were done in MATLAB (Mathworks, Natick, MA).

In the first step, the composition of eluent B was optimized. This was done in two different blocks, the first was a screening block where the important factors were determined *via* a highly reduced factorial design (Plackett-Burman). The design and experimental domain are shown in Table 1. The eight chromatograms, conditions given by each row in Table 1, were evaluated quantitively and ranked using a multivariate ranking function, where optimal chromatographic conditions are attained at the minimum of the function. The chromatographic resolution statistic (CRS) function takes peak resolution, peak distribution and total analysis time into account, thus enabling an objective and quantitative ranking (23).

In the second block of the first step, the best chromatogram from the first block was used as the basis for a new experimental design (Table 1, row 6). The experimental factors identified as influential in the first block were varied according to a fractional factorial design (2^{4-1}) shown in Table 2. These chromatograms were also evaluated and ranked according to the above described procedure.

Response surfaces of the experimental domain were developed in two different ways, either by fitting a linear

TABLE 2

Reduced Factorial Design of the Second[a] Experimental Block

Run number	EtOH (vol%)	Isooctane (vol%)	Gradient[b] profile	B% endpoint	CRS[c] value
1	40	10	−2	75	13.775
2	50	10	−2	75	19.133
3	50	10	+2	75	12.099
4	40	20	+2	75	9.910
5	50	10	−2	100	12.416
6	40	20	−2	100	12.418
7	50	10	+2	100	11.688
8	50	20	+2	100	10.022

[a]Chosen from a full factorial (Ref. 24).
[b]Gradient: A to B in 80 min.
[c]CRS, chromatographic resolution statistic.

polynomial function in the experimental factors to the corresponding CRS values and plotting two factors at a time, or by developing a PLS regression model relating the experimental factors to the resolution and peak asymmetry factors and the total analysis time. These response surfaces enabled location of optimal experimental conditions for the B eluent.

The optimal A eluent composition was obtained in the second step by varying the content of isooctane, *i.e.*, altering the elution strength of the solvent system.

Identification. The TAG molecular species were identified on the basis of their ECN values. These were determined using the Snyder's polarity index values (P') (24), calculated for the solvent system composition at the time of elution of the set of TAG standards. The ECN values were determined by fitting a fifth degree polynomial in the calculated P' to the known ECN values of a set of saturated TAG standards, where

$$ECN_i = \beta_0 + \beta_1 P'_i + \beta_2 P'^2_i + \beta_3 P'^3_i + \beta_4 P'^4_i + \beta_5 P'^5_i \quad [1]$$

and the vector of regression coefficients was found by matrix least squares

$$\beta = (X'X)^{-1}X'Y \quad [2]$$

where Y is the vector of ECN values of the saturated set of nine TAG standards and the X-matrix is the right-hand side of the ECN equation. Thereafter the ECN values were predicted for unknown TAG molecular species. The relative prediction (PRED) error (E_R) for each standard (STD) species was calculated as

$$E_R = (ECN_{STD} - ECN_{PRED})/ECN_{STD} \quad [3]$$

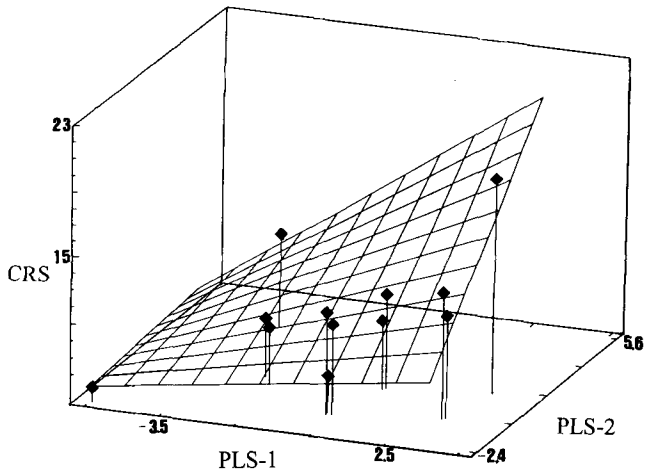

FIG. 1. Partial least squares correlations in latent variables (PLS) response surface plot, chromatographic resolution statistic (CRS) value as a function of the first and second linear combinations of the original experimental variables.

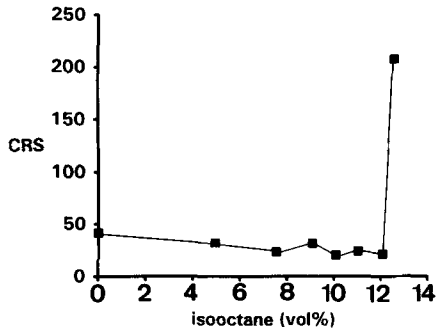

FIG. 2. Chromatographic resolution statistic (CRS) value as a function of isooctane level in the A eluent.

RESULTS AND DISCUSSION

The results of the optimization can be seen in Figures 1 and 2. The experimental domain was mapped by response-surface modelling and PLS response surface modelling which thus enabled the graphical localization of optimal chromatographic conditions. The PLS-1 and PLS-2 axes are the linear combinations of the original experimental variables, the so-called latent factors, which have maximal linear correlation to the response variable, the CRS values (Fig. 1). In this plot the location of each experiment on the response surface can be seen (Fig. 1). As stated above, optimal chromatography is attained where the CRS function shows a minimum, in this figure at the bottom left corner (Fig. 1). The correlations between the original experimental variables and the latent factors can be utilized to elucidate the relation between the chromatographic conditions and the quality of the chromatography, expressed as the CRS value. The response surfaces can be compared with one another, in order to graphically locate experimental conditions at the minimum of the function, thus enabling optimization of chromatographic conditions. Optimal values of the chromatographic conditions can also be directly predicted using PLS regression. These were from acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol), at a column temperature of 50°C, a flowrate of 1.5 mL/min and a gradient of 100% A to 100% B in 80 min with a negative exponential profile.

The results from the optimization are strongly dependent on the optimization criteria; different chromatographic systems can be obtained by altering the goal of the optimization. In order to develop a system showing maximal separation within the explored domain, with no regard to the total analysis time, the resolution factors can be emphasized in the PLS regression. The system predicted to give maximal resolution (within the explored domain) had the composition of acetonitrile to acetonitrile/ethanol (59:41, vol/vol), at a column temperature of 50°C and a flowrate of 1.5 mL/min.

Some general remarks can be made based on Table 1. A lower elution temperature causes a decrease in the CRS values. Temperature was subsequently held at 50°C in the second set of experiments. The flowrate was also held constant at 1.5 mL/min in order to reduce the number of variables. Increasing the amount of isooctane in eluent A up to 12% (Fig. 2) was shown to yield lower CRS values. The final analysis time was shown to be strongly

dependent on the amount of isooctane in both eluent A and B.

Retention indices such as the ECN concept have found widespread use in lipid analysis, especially for the identification of molecular species of TAG (2,16,17). In the ECN concept, a mathematical model is built between the retention times of the TAG molecular species and a weighted sum of the total number of carbon atoms and the number of double bonds in the fatty acyl chains present in the TAG molecule. The most common mathematical model used is a logarithmic relation (4,9). The results have been used to provide a means of identifying TAG molecular species and also to predict the chainlength and number of double bonds (4,9,17,18).

Different nomenclatures are used by workers in this field. In the present study we will restrict ourselves to the nomenclature used by Podlaha and Töregård (9). Our choice of using a polynomial expansion to model the data was motivated by the fact that we use gradient elution. Any nonlinearity in the data due to the solvent gradient would therefore be properly handled by choosing the appropriate degree of the polynomial. A fifth degree polynomial model was found to give the lowest relative prediction error (approximately 1%). We also used P′ (Snyders polarity index; Ref. 24) instead of the retention times in the ECN model in order to compensate for different gradient profiles. The P′-value is a measure of the chromatographic environment experienced by each individual molecular species at the time of elution and can thus be considered as a more pertinent description of the chemical characteristics of the molecule. The P′-value for the solvent mixture at the time of elution can be estimated by calculating the amounts of the different solvents present in the mobile phase and by using the volume fractions as coefficients in a simple linear function of each pure solvent P′ (24). Further, our use of the ECN values is restricted to simply obtaining an identification or an ordering number for each of the separated peaks. The results can thus be compared to virtually any RP-HPLC system just by employing a set of saturated TAG standards to build the ECN model.

Prediction of the ECN values for molecular species was done for a range of different natural oils (Table 3). Molecular species that are not determined in this chromatographic system are placed in the expected elution order (25,26). The separation potential of the system can be described by comparing the number of carbons and the degree of unsaturation in the fatty acid moieties, *i.e.*, the partition number, PN, to the observed P′ differences of different TAG, a selection of which are listed in Table 4.

TABLE 3

ECN Values of Molecular Species[a]

Species[b]	PN	ECN	P′[c]
DDD	30	30.00	4.70
LnLnLn	36	35.24	4.45
LaLaLa	36	36.00	4.41
LLnLn	38	37.06	4.36
LLLn	40	39.13	4.27
LnLnO	40	39.13	4.27
LnLnP	40	39.61	4.25
LLL	42	40.79	4.20
LLnO	42	41.03	4.19
LLnP	42	41.27	4.18
LnLnSt	42	n.d.[d]	
MMM	42	42.01	4.15
LLO	44	42.79	4.12
LnOO	44	42.79	4.12
LLP	44	43.06	4.11
LLnSt	44	n.d.	
LnOP	44	43.06	4.11
LnPP	44	n.d.	
LOO	46	44.63	4.06
LLSt	46	n.d.	
PdPdPd	45	45.01	4.05
LOP	46	45.01	4.05
LnOSt	46	n.d.	
LPP	46	45.40	4.04
LnPSt	46	n.d.	
OOO	48	46.77	4.01
LOSt	48	n.d.	
OOP	48	47.29	4.00
LPSt	48	n.d.	
OPP	48	47.87	3.99
PPP	48	48.49	3.98
LnStSt	48	n.d.	
OOSt	50	49.16	3.97
LStSt	50	49.16	3.97
OPSt	50	49.89	3.96
PPSt	50	50.68	3.95
HdHdHd	51	51.54	3.94
OStSt	52	52.47	3.93
PStSt	52	53.49	3.92
StStSt	54	54.59	3.91
NdNdNd	57	57.08	3.89
AdAdAd	60	60.02	3.87

[a]Abbreviations: Ad, arachidonic acid; D, decanoic acid; PN, partition number; ECN, equivalent carbon number; Hd, heptadecanoic acid; Ln, linolenic acid; La, lauric acid; L, linoleic acid; Nd, nonadeanoic acid; O, oleic acid; P, palmitic acid; Pd, pentadecanoic acid; St, stearic acid.

[b]Fatty acids given in arbitrary order in regard to the glycerol backbone.

[c]Calculations not corrected for system void volume.

[d]n.d., Not determined, species given in the expected elution order according to References 25 and 26.

TABLE 4

P′ Relation to Partition Number (PN)

PN difference in TAG	Example of fatty acid substitution[a]	P′ difference
0	P replaced with O	0.02–0.01
2	P replaced with L	0.11–0.06
2	O relaced with L	0.09–0.05
2	L replaced with Ln	0.09–0.06
2	St replaced with O	0.10–0.02
2	St replaced with P	0.08–0.01
4	P replaced with Ln	0.20–0.12
4	O replaced with Ln	0.18–0.11
4	St replaced with L	0.19–0.06
6	St replaced with Ln	0.28–0.12

[a]Abbreviations: TAG, triacylglycerols; see Table 3 for other abbreviations.

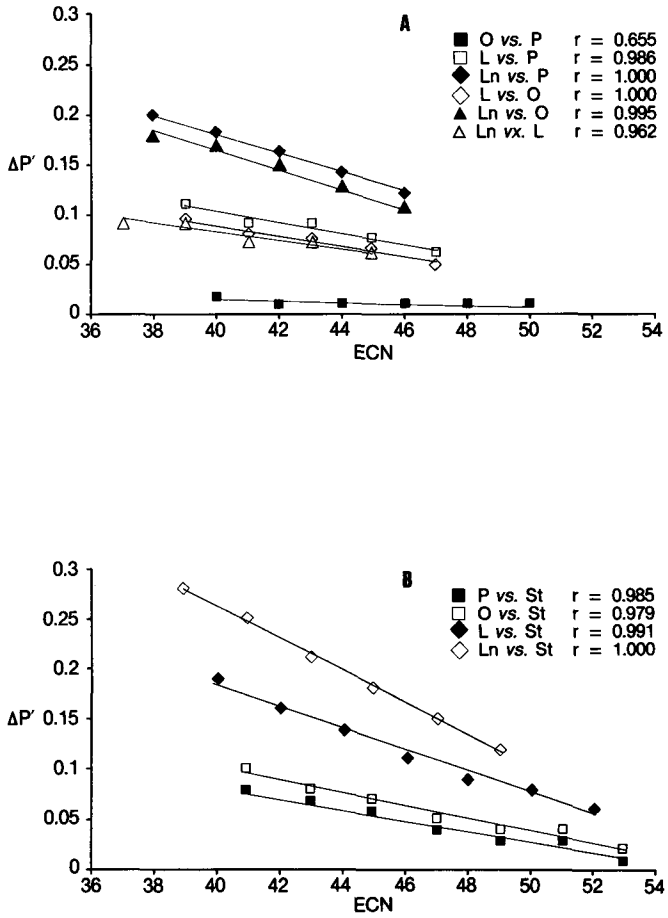

FIG. 3. P′ difference as a function of average equivalent carbon number (ECN) of (A) triacylglycerol (TAG) molecular species and (B) TAG molecular species containing stearic acid. Abbreviations: O, oleic; P, palmitic; L, linoleic; Ln, linolenic.

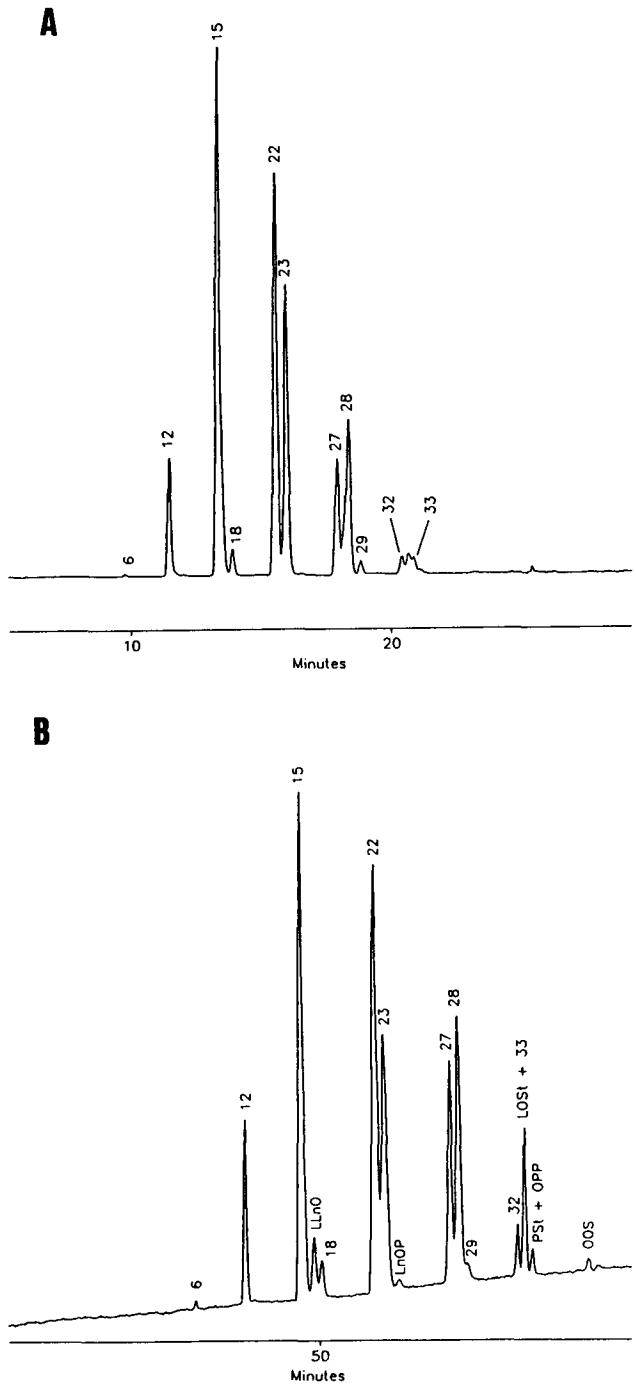

FIG. 4. Reversed-phase high-performance liquid chromatograms for soybean oil. Solvent system for (A): acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol), and for (B): acetonitrile to acetonitrile/ethanol (59:41, vol/vol). Conditions: see text. Peak numbering refers to Table 4. See Figure 3 for abbreviations.

Plots of these (Fig. 3) show decreasing trends when moving from low ECN values (early-eluting) to high values (late-eluting). Species that will coelute in this system, provided they coexist in the same oil, can be seen in this plot where the lines intersect (Fig. 3B).

The number of RP-HPLC distinguishable molecular species (not considering positional or optical isomers) is given by $(n^3 + 3n^2 + 2n)/6$, where n is the number of fatty acids (27). This number is the upper limiting value, the random distribution. The number of species found in most biosystems is much lower. Also, many of the species included in this number are probably present at levels below the detection limit (100 ng). Soybean oil (Fig. 4A) with 6 major fatty acids can contain at the most 56 molecular species (random distribution); 12 peaks are separated and 11 are identified, these are listed in Table 5. By changing the optimization criteria as mentioned above to emphasize separation at the expense of total analysis time, the average resolution can be enhanced from 3.9 to 4.4 (Fig. 4A), thus separating 16 peaks (Fig. 4B). Rapeseed oil (Fig. 5A) has 5 major fatty acids and can exhibit 35 molecular species at the most according to the random distribution, 12 are separated and identified in this chromatogram (Fig. 5A, Table 5). Palm oil (Fig. 5B) also has 5 major fatty acids as does linseed oil (Fig. 6A), 11 and 13 peaks are separated and identified, respectively (Figs. 5B, 6A, Table 5). Tallow has a more complex fatty acid composition with 9 major fatty acids (Fig. 6B), 165 random combinations are possible while 16 peaks are separated and 12 are identified (Fig. 6B, Table 6). The decidedly more complex fish oil, a commercial commodity (Fig. 7), has a fatty acid com-

TABLE 5

ECN of Oils from Different Sources[a]

Peak number	P′[b]	ECN value	Soybean Area %	Soybean Species	Rapeseed Area %	Rapeseed Species	Palm Area %	Palm Species	Linseed Area %	Linseed Species
1	4.70	30.00								
2	4.45	35.24							34.7	LnLnLn
3	4.43	35.61								
4	4.41	36.00								
5	4.36	37.06							14.0	LLnLn
6	4.35	37.28	0.2	LLnLn						
7	4.34	37.50								
8	4.33	37.73								
9	4.31	37.20								
10	4.30	38.43								
11	4.28	38.90								
12	4.27	39.13	5.6	LLLn	0.8	LLLn			19.3	LnLnO
13	4.25	39.61							5.2	LnLnP
14	4.22	40.32								
15	4.20	40.79	32.3	LLL	5.1	LLL				
16	4.19	41.03			0.4	LLnO			5.7	LLnO
17	4.18	41.27							6.5	LLnP
18	4.17	41.51	1.4	LLnP						
19	4.15	42.01								
20	4.14	42.26								
21	4.14	42.26								
21	4.13	42.52								
22	4.12	42.79	23.4	LLO	18.9	LLO/LnOO[c]			6.4	LnOO
23	4.11	43.06	17.0	LLP	0.4	LLP/LnOP[d]	0.6	LLP	3.8	LnOP
24	4.10	43.35								
25	4.09	43.65								
26	4.08	43.96								
27	4.06	44.6	36.9	LOO	26.8	LOO	0.6	LOO	0.7	LOO
28	4.05	45.01	1.5	LOP	2.0	LOP	7.5	LOP	2.2	LOP
29	4.04	45.40	0.7	LPP			7.5	LPP		
30	4.03	45.82								
31	4.02	46.28								
32	4.01	46.77	0.6	OOO	41.4	OOO	2.3	OOO	0.8	OOO
33	4.00	47.29	0.4	OOP	2.1	OOP	29.2	OOP	0.4	OOP
34	3.99	47.87					42.8	OPP		
35	3.98	48.49			0.4	OPP	4.6	PPP		
36	3.97	49.16			0.8	OOSt	1.1	OOSt	0.3	OOSt
37	3.96	49.89					3.5	OPSt		
38	3.95	50.68					0.3	PPSt		
39	3.94	51.54								
40	3.93	52.47								
41	3.92	53.49								
42	3.91	54.59								
43	3.89	57.08								
44	3.87	60.02								
Total area%:			100.0		99.1		100.0		100.0	

[a]Abbreviations: See Table 3.
[b]Calculations not corrected for system void volume.
[c]The species LLO (0.9%) and LnOO do not coelute, as can be seen in Figure 5A, but have the same P′ value in this example of rapeseed oil.
[d]The species LLP and LnOP coelute.

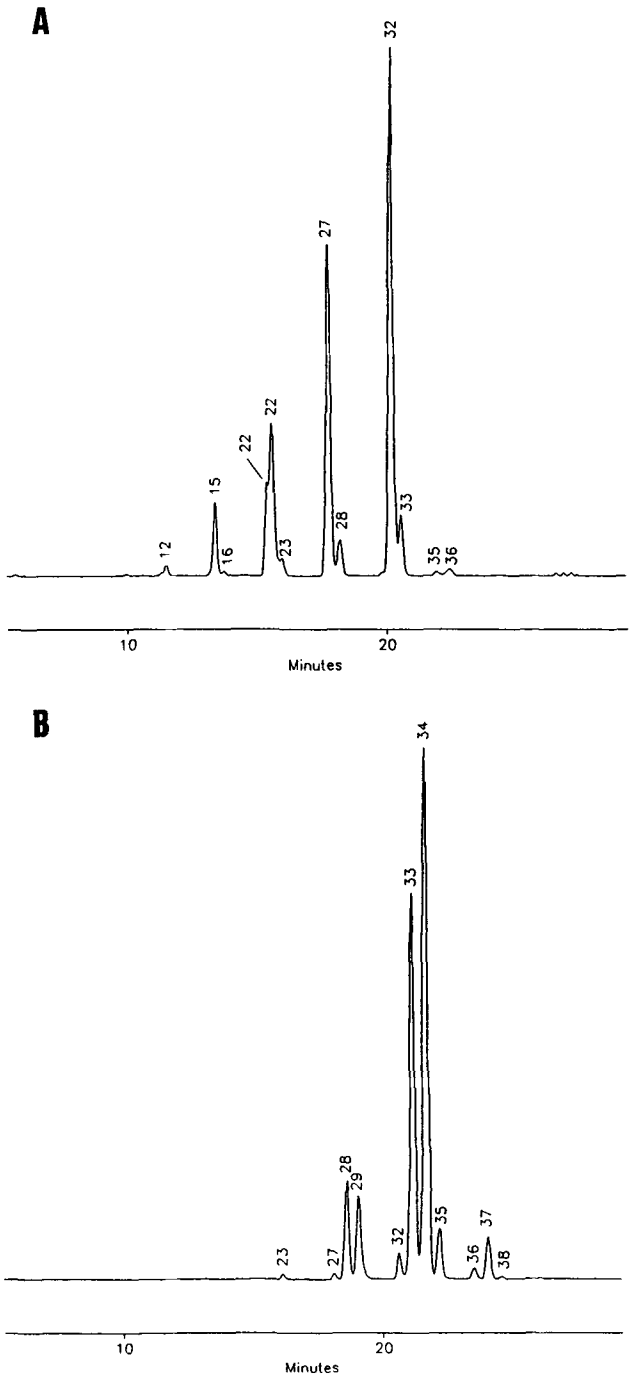

FIG. 5. Reversed-phase high-performance liquid chromatograms for (A) rapeseed oil and (B) palm oil. Solvent system: acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol). Conditions: see text. Peak numbering refers to Table 4.

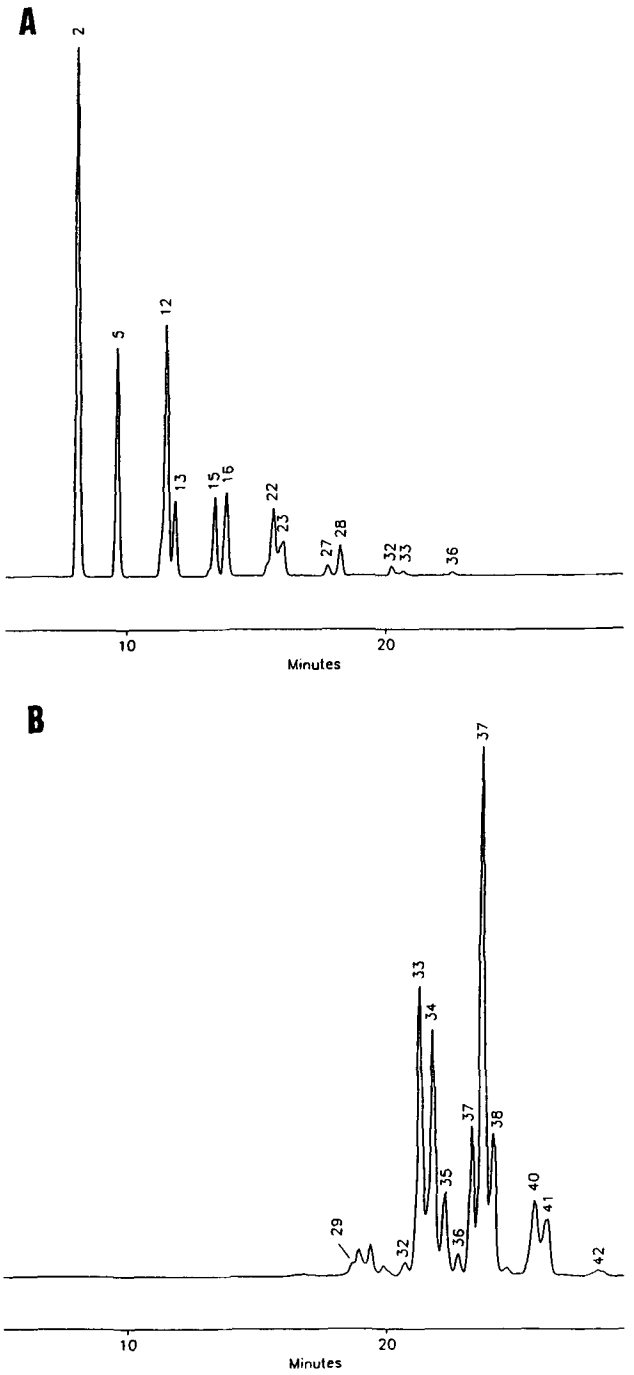

FIG. 6. Reversed-phase high-performance liquid chromatograms for (A) linseed oil and (B) tallow. Solvent system: acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol). Conditions: see text. Peak numbering refers to Tables 4 and 5, respectively.

TABLE 6

ECN of Oils from Different Sources[a]

Peak number	P'[b]	ECN value	Fish Area %	Fish Species	Tallow Area %	Tallow Species	Standard Area %	Standard Species
1	4.70	30.00					9.8	DDD
2	4.45	35.24	2.3					
3	4.43	35.61	3.3					
4	4.41	36.00					10.9	LaLaLa
5	4.36	37.06						
6	4.35	37.28	3.5					
7	4.34	37.50	7.9					
8	4.33	37.73	4.5					
9	4.31	37.20	1.9					
10	4.30	38.43	2.7					
11	4.28	38.90	8.7					
12	4.27	39.13	1.0					
13	4.25	39.61						
14	4.22	40.32	1.4					
15	4.20	40.79	14.3					
16	4.19	41.03	7.3					
17	4.18	41.27	0.8					
18	4.17	41.51						
19	4.15	42.01	0.9				11.4	MMM
20	4.14	42.26	1.1					
21	4.13	42.52	13.8					
22	4.12	42.79	7.4					
23	4.11	43.06	2.1					
24	4.10	43.35						
25	4.09	43.65	2.7					
26	4.08	43.96	2.3					
27	4.06	44.63						
28	4.05	45.01					16.8	PdPdPd
29	4.04	45.40	3.6		2.2	LPP		
30	4.03	45.82	2.1		1.9			
31	4.02	46.28			0.7			
32	4.01	46.77			0.8	OOO		
33	4.00	47.29			16.4	OOP		
34	3.99	47.87	2.5		15.1	OPP	11.6	PPP
35	3.98	48.49	0.9		5.0	PPP		
36	3.97	49.16			1.1	LStSt		
37	3.96	49.89			40.6	OOSt/OPSt[c]		
38	3.95	50.68			4.8	PPSt		
39	3.94	51.54			0.4		23.2	HdHdHd
40	3.93	52.47			5.6	OStSt		
41	3.92	53.49			4.5	PStSt	12.1	StStSt
42	3.91	54.59			0.7	StStSt		
43	3.89	57.08					2.1	NdNdNd
44	3.87	60.02					2.1	AdAdAd
Total area%:			99.0		99.8		100.0	

[a]Abbreviations: See Table 3.
[b]Calculations not corrected for system void volume.
[c]The species OOSt (7.2%) and OPSt do not coelute, as can be seen in Figure 6B, but have the same P' value in this sample of tallow.

position of 15 major fatty acids with 680 possible random combinations. This oil contains molecular species of quite a different nature than those present in seed oils, due to the high content of polyunsaturated fatty acids. However, in less than 25 min some 46 species are separated, of which 24 are major (>0.8 area %) (Fig. 7, Table 6).

In conclusion, we have formally optimized an RP-HPLC method for the separation of TAG molecular species using statistical experimental design and multivariate optimization. This strategy enables great flexibility in the development process to tailor the system after desired specifications, limited only by the chosen experimental domain. The system thus developed afforded excellent resolution within a short total analysis time, which was exemplified on several examples of natural seed oils, tallow and fish oil. Identification of the TAG species was done utilizing the ECN concept with Snyders P' at the time of elution instead of retention times. This concept was shown, through Δ P'-ECN plots, to furnish a means of detecting potentially critical pairs of molecular species present in different oils.

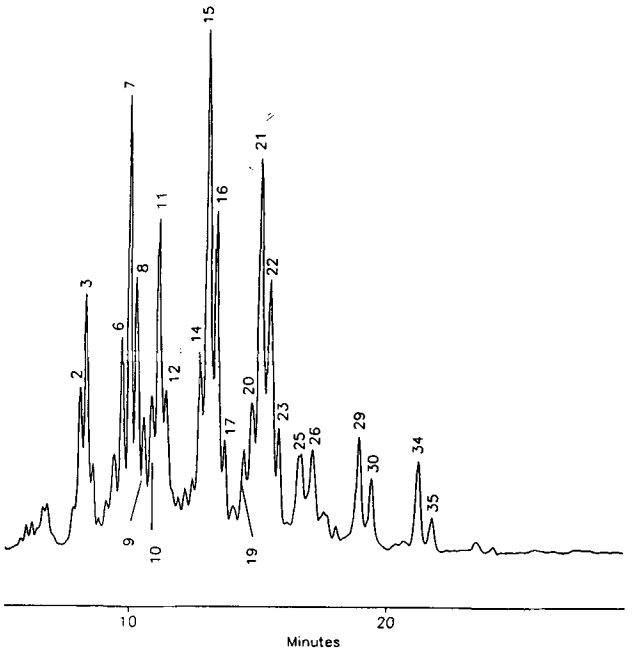

FIG. 7. Reversed-phase high-performance liquid chromatogram for fish oil. Solvent system: acetonitrile/isooctane (90:10, vol/vol) to acetonitrile/ethanol/isooctane (40:35:25, by vol). Conditions: see text. Peak numbering refers to Table 5.

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ARTICLE

Maternally-Supplied Fish Oil Alters Piglet Immune Cell Fatty Acid Profile and Eicosanoid Production[1]

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This study was designed to examine the incorporation of omega-3 (n-3) fatty acids into the immune tissues of pigs nursing fish oil-fed sows and to determine the effect of maternal dietary n-3 consumption on *in vitro* immune cell eicosanoid production. On day 107 of gestation, 12 sows were randomly allotted to a diet containing either 7% menhaden fish oil (MFO) or lard (LRD). The fatty acid profile of serum, liver, thymus, splenocytes and alveolar macrophages (AM) of 18–21-day-old pigs was significantly affected by the fat source provided to the sow. Arachidonic acid (20:4n-6) content was typically reduced by more than 50% in MFO as compared with LRD pigs. In MFO pigs, eicosapentaenoic acid (20:5n-3) was the major n-3 polyunsaturated fatty acid, and its levels matched or exceeded those of arachidonic acid. Basal release of prostaglandin E, thromboxane B and leukotriene B by AM was 60–70% lower in MFO *vs.* LRD pigs. However, when these immune cells were stimulated with calcium ionophore A23187, release of leukotriene B was similar in MFO and LRD pigs. In conclusion, substituting MFO for LRD in a sow's late-gestation and lactation diet greatly elevated the content of n-3 fatty acids in the nursing pig immune cells and generally reduced *in vitro* eicosanoid release by pig immune cells.
***Lipids* 28, 677–682 (1993).**

Apart from their role as storage and transport forms of metabolic fuel, the fatty acid portion of dietary fats serves several other important functions in the body. These include providing for the essential fatty acid requirements, acting as structural components of cell membranes and serving as precursors for eicosanoid production. Eicosanoids, such as prostaglandins and leukotrienes, are recognized as important modulators of humoral and cell-mediated immune responses (1,2). Considerable evidence has accumulated which demonstrates that dietary fats influence the immune response, partly through their ability to modulate eicosanoid production (3). The fatty acid moieties of phospholipids determine many membrane functions. The possible influence of lipids on receptor binding, signal transduction and lymphocyte proliferation makes knowing the fatty acid composition of immune cells of particular interest (4,5).

With the increased interest in providing premature infants and neonates lipid emulsions or infant formulas enriched with n-3 polyunsaturated fatty acids (PUFA), it is important that we understand the potential impact of these lipids on immune tissues in this age group. Little is known about the influence that maternal dietary fat source has on the fatty acid composition and eicosanoid production of neonatal immune cells. The use of swine in biomedical research has been growing steadily. Miller and Ullrey (6) have described some of the advantages of using the pig as a model for human nutrition research. Previous studies in this laboratory demonstrated that the inclusion of fish oils in sow diets during late-gestation and lactation significantly elevated n-3 PUFA levels in the blood of nursing pigs (7).

The objectives of this study were: (i) to determine the incorporation of n-3 PUFA into the immune cells of pigs nursing fish oil-fed sows and (ii) to quantitate the impact of n-3 PUFA incorporation on eicosanoid production by pig immune cells.

MATERIALS AND METHODS

Animals and diets. On day 107 of gestation, 12 crossbred sows (Landrace × Duroc, Columbia, MO) were randomly allotted to one of two diets, in which menhaden fish oil (MFO) was substituted for lard (LRD) at 7% by weight of the diet. Diets were isocaloric and formulated to meet National Research Council requirements (8). See Table 1

TABLE 1

Composition of Experimental Diets Fed to Sows

Ingredient	Dietary treatment groups	
	LRD (g/100 g)	MFO (g/100 g)
Ground corn	58.9	58.9
Oats	10.1	10.1
Soybean meal (44% crude protein)	20.0	20.0
Lard (LRD)[a]	7.0	—
Menhaden fish oil (MFO)[b]	—	7.0
Dicalcium phosphate	2.7	2.7
Vitamin mix[c]	0.5	0.5
Trace mineral salt mix[d]	0.5	0.5
Ground limestone	0.4	0.4

[a]Analyzed fatty acid composition (wt%): 14:0, 1.0; 16:0, 25.4; 16:1n-7, 2.8; 18:0, 13.7; 18:1n-9, 42.3; 18:2n-6, 10.8; 18:3n-3, 0.7.
[b]Courtesy of Zapata Haynie Corp. (Reedville, VA) with an analyzed fatty acid composition of (wt%): 14:0, 6.4; 16:0, 16.6; 16:1n-7, 10.4; 18:0, 2.6; 18:1n-9, 13.9; 18:2n-6, 1.5; 18:3n-3, 1.2; 20:1, 2.6; 20:5n-3, 14.3; 22:1, 1.8; 22:5n-3, 2.2; 22:6n-3, 12.6. Fish oil was stabilized against autooxidation by the addition of 500 ppm of ethoxyquin.
[c]Supplied per kg of diet: 5,506 IU vitamin A, 550 IU vitamin D_3, 27 IU vitamin E, 5.5 mg riboflavin, 27 mg *d*-pantothenic acid, 33 mg niacin, 2.2 mg thiamin, 2.2 mg vitamin B_6, 1.1 mg folic acid, 669 mg choline, 0.038 mg vitamin B_{12}, 2.2 mg menadione and 0.165 mg biotin.
[d]Supplied in mg per kg of diet: 125 Zn, 125 Fe, 25 Mn, 15 Cu, 0.5 I, 0.1 Se, 4,258 NaCl.

[1]Presented in part at the 1991 Midwest Animal Sciences meeting, Des Moines, Iowa.
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Abbreviations: AA, arachidonic acid; AM, alveolar macrophage; EDTA, ethylenediaminetetraacetate; EPA, eicosapentaenoic acid; HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; LRD, lard; LTB, leukotriene B; MEM, Minimal Essential Medium; MFO, menhaden fish oil; PGE, prostaglandin E; PUFA, polyunsaturated fatty acids; TXB, thromboxane B.

for complete composition. Sows were individually fed 1.8–2.1 kg of experimental diets daily from day 107 of gestation until farrowing. An additional 0.45 kg was fed to each sow per day for each suckling pig during the 28-d lactation period. Additional details regarding housing and handling of animals is described elsewhere (7). Housing, handling and sample collection procedures conformed to policies and recommendations of the University of Missouri's Laboratory Animal Care Advisory Committee.

Sample collection. At 3–4 wk of age, one pig from each litter (n = 6) was killed for tissue collection. The pigs were anesthetized with ketamine (20 mg/kg) and acepromazine (1 mg/kg). Blood (20 mL) was collected by venipuncture and left to clot for subsequent serum isolation. A portion of the spleen was removed and placed on ice in a 50-mL tube filled with sterile-filtered ice-cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (UMC Immunobiology Core Facility, Columbia, MO) for subsequent splenocyte isolation. Single cell suspensions of splenocytes were made by forcing the spleen sample through a tissue sieve (Sigma, St. Louis, MO) 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. Red blood cells and dead cells were removed by centrifugation of the spleen cell suspension over lymphocyte separation medium, density 1.077–1.080 g/mL at 20°C, as described by the manufacturer (Organon Teknika Corp., Durham, NC). Mononuclear cells at the interface (predominantly lymphocytes and monocytes) were collected, washed twice and stored in 1 mL 10 mM ethylenediaminetetraacetate (EDTA). Samples of the liver and thymus were snap frozen in 20-mL glass vials using a dry ice-acetone bath. All samples were stored at −80°C until lipid analysis.

Alveolar macrophage (AM) and peritoneal cell isolation and eicosanoid production. Resident peritoneal cells were collected by filling the peritoneal cavity with ≈400 mL of sterile ice-cold Hank's. The abdomen was gently massaged, then fluid was removed with a 60-mL syringe. Total recovery averaged ≈200 mL. For the collection of AM, the trachea was clamped shut and the lungs were excised. The lungs were filled with 60 mL of ice-cold Hank's and gently shaken. The contents of the lungs were filtered through sterile gauze into 50-mL tubes on ice. This process was repeated twice (total recovery was ≈100 mL).

The tubes containing the AM or resident peritoneal cells were spun down (500 × *g* for 15 min) and washed twice with Minimal Essential Medium (MEM) with 2 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES), penicillin (100,000 U/L) and streptomycin (0.17 mmol/L), pH 7.4 (MEM). The cells were then spun at 500 × *g* for 10 min and resuspended in 10 mL of MEM. Cells were counted electronically (Coulter Electronics, Inc., Hialeah, FL) and resuspended at a final concentration of 5×10^6 cell/mL of MEM. The AM were more than 95% macrophages based on morphology, Wright-Giemsa staining (9) and adherence properties. The resident peritoneal cell preparations were 60–70% macrophages based on the same criteria.

To each well of a 24-well cell culture plate, 0.5 mL (2.5×10^6) of the AM or peritoneal cell suspension was added in addition to 0.5 mL of MEM with 10% autologous serum. Plates were placed in an incubator at 37°C with 5% CO_2 for 4 h to allow cells to adhere. After washing the adhered cells three times with MEM, 0.5 mL of MEM with 10% autologous serum was added to each well. Then 0.5 mL/well of MEM alone (controls) or containing lipopolysaccharide from *E. coli* O111:B4 (5 μg/mL) or calcium ionophore A23187 (2 μg/mL) was added to wells in order to stimulate eicosanoid production. At the times indicated, supernatants were collected in 1.5-mL polypropylene microcentrifuge tubes, spun (10,000 rpm, 3 min), then transferred into a new tube and stored at −80°C.

Lipid extraction and analysis. Lipids were extracted from the serum, liver, thymus, splenocytes and AM following the Folch method as described elsewhere (10). Methyl esters of fatty acids were prepared by transmethylation using 4% H_2SO_4 in methanol. Fatty acid methyl esters were identified using a Hewlett-Packard gas chromatograph (Sunnyvale, CA) Model 5890A with a 30 m capillary column (Supelcowax 10, Supelco, Bellefonte, PA). Results, expressed as percent of total fatty acids, were determined using a Hewlett-Packard 3396A integrator.

Eicosanoid analysis. The concentration of prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2) and leukotriene B_4 (LTB_4) in the cell supernatants was determined using commercially available enzyme immunoassay kits for each eicosanoid as described by the manufacturer (Advanced Magnetics Inc., Boston, MA). Information on the cross-reactivity of these antisera with eicosapentaenoic acid (EPA)-derived metabolites (*e.g.*, PGE_3, TXB_3 and LTB_5) was not available from the manufacturer. Therefore results are expressed as micrograms of PGE or LTB per liter of supernatant. All cell supernatants were assayed in duplicate. When necessary, supernatants were diluted with MEM prior to analysis. The linear portion of a typical standard curve ranged from 20.0 to 0.027 ng/mL for PGE and 10.0 to 0.014 ng/mL for TXB and LTB, respectively.

Statistical analysis. Fatty acid data were subjected to one-way analysis of variance (ANOVA) to test for an effect of fat source. Eicosanoid (*i.e.*, PGE, LTB) data were log transformed to achieve homogeneity of variance then analyzed by ANOVA. All analyses were conducted on a Macintosh II computer using version 1.03 of StatView II (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Fatty acid composition. The replacement of LRD with MFO in the late gestation and lactation diet of sows significantly modified the fatty acid profile of the serum, liver and immune tissues of nursing pigs (Tables 2 and 3). The two fat sources differed significantly in the amount of saturated fatty acids and PUFA they contained. However, there was little difference in the total saturated fatty acid content in the serum or tissues examined. Total PUFA content in the serum and liver, but not immune tissues, was altered by dietary fat source. Oleic acid (18:1n-9) was the major monounsaturated fatty acid in both diets and in the samples analyzed. The level of 18:1n-9, as well as the total monounsaturated fatty acids, were higher ($P < .05$; Tables 2 and 3) in the serum, liver, thymus and alveolar macrophages from the LRD pigs as compared with the MFO pigs. These differences were a reflection of dietary oleic acid and total monounsaturated fatty acid content of the diets.

The relative levels of individual and total n-6 and n-3 PUFA in the pigs were significantly altered by the source

TABLE 2

Mean Fatty Acid Content of Total Serum and Liver Fatty Acids from Three-Weeks-Old Piglets Nursing Sows Fed Diets Containing 7% (by wt) Either Lard (LRD) or Menhaden Fish Oil (MFO)[a]

	Sample source					
	Serum			Liver		
	Maternal fat source					
Fatty acid[b]	LRD[c]	MFO		LRD	MFO	
	(g/100 g total fatty acids)					
14:0	0.7	0.8	0.2[d]	0.5	0.4	0.01[d]
16:0	22.9	22.3	1.3	17.9	18.0	0.8
16:1	4.2	3.6	0.4	2.7	2.0[e]	0.2
18:0	7.5	5.3	0.8	13.8	15.4	1.2
18:1n-9	22.5	13.6[f]	1.8	12.2	6.9[f]	0.9
18:1n-7	2.5	1.8[f]	0.1	2.8	1.6[f]	0.1
18:2n-6	23.0	19.1[e]	1.1	14.4	10.3[e]	1.0
20:3n-6	trace	trace		0.7	0.5	0.1
20:4n-6	9.3	4.3[f]	0.7	22.4	9.0[g]	1.1
20:5n-3	0.1	15.2[g]	1.6	0.2	11.9[g]	1.0
22:4n-6	0.6	0.0[g]	0.1	1.1	0.1[g]	0.1
22:5n-6	trace	trace		1.0	0.1[g]	0.01
22:5n-3	0.7	1.7[f]	0.2	1.9	3.6[g]	0.1
22:6n-3	1.6	7.4[f]	0.8	4.7	16.5[g]	1.0
Total SAT[h]	31.1	28.4	1.6	32.2	33.8	1.4
Total MONO[i]	29.2	19.0[f]	1.7	17.7	10.5[g]	0.8
Total n-6 PUFA[j]	34.0	24.0[g]	1.3	39.9	20.1[g]	2.1
Total n-3 PUFA[k]	2.8	25.5[g]	2.4	6.9	32.3[g]	1.8
Total PUFA[l]	37.1	49.5[f]	2.0	47.0	52.5[e]	1.6

[a]Sows were fed diets containing 7% (by wt) LRD or MFO for 1 wk prior to farrowing. Piglets nursed sows within the same treatment group. Samples were collected from piglets upon weaning (3 wk).
[b]Fatty acids are denoted by the number of carbons: number of double bonds, followed by the position of the first double bond relative to the methyl-end (n-) of the fatty acid.
[c]Values are expressed as means (n = 6) for fatty acids that accounted for 0.7% or more of the total fatty acids present in the sample; trace, less than 0.3% of the total fatty acids.
[d]Pooled SEM.
[e]Mean MFO value significantly different from LRD value ($P < 0.05$).
[f]Mean MFO value significantly different from LRD value ($P < 0.005$).
[g]Mean MFO value significantly different from LRD value ($P < 0.0005$).
[h]Sum total area percentage of 14:0, 16:0 and 18:0. SAT, saturated.
[i]Sum total area percentage of 16:1, 18:1n-9, 18:1n-7 and 20:1. MONO, monounsaturated.
[j]Sum total area percentage of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. PUFA, polyunsaturated fatty acids.
[k]Sum total area percentage of 18:3n-3, 18:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.
[l]Sum total area percentage of those fatty acids listed in footnotes j and k.

of fat fed to the sow. Feeding fish oil to the sows lead to an elevation of n-3 PUFA with a concomitant decrease in n-6 PUFA. For example, arachidonic acid (AA; 20:4n-6) levels in the serum, liver and immune tissues were decreased by more than 50% ($P < 0.005$; Tables 2 and 3) in MFO pigs as compared to LRD pigs. The levels of the other major n-6 PUFA, linoleic acid (18:2n-6), were only affected modestly ($P < 0.05$; Tables 2 and 3). In fact, 18:2n-6 levels in the immune tissues were not significantly different between LRD and MFO pigs.

In LRD pigs, EPA (20:5n-3) was found only in trace amounts (<0.5%). Feeding MFO to the sows greatly increased the amount of this n-3 PUFA in the serum, liver, and immune tissues of pigs ($P < 0.0005$). In the serum, liver, splenocytes and alveolar macrophages of the MFO pigs, the level of EPA exceeded that of AA. The levels of other n-3 PUFA, 22:5n-3 and 22:6n-3, were also elevated in those pigs suckling MFO-fed sows. Total n-3 PUFA content of the serum and tissues sampled accounted for between 7–17% of the total PUFA in LRD pigs, while in MFO pigs they accounted for between 46–65% of the total PUFA. However, total PUFA levels in the thymus, splenocytes and AM did not significantly differ between LRD and MFO pigs.

Eicosanoid production. The impact of maternal dietary fat source on piglet immune cells PGE production is shown in Table 4. There was a 12-fold reduction in PGE release over a 24-h period by unstimulated (basal) AM isolated from the MFO-piglets as compared to the LRD piglets. Co-culturing piglet AM with either calcium ionophore or endotoxin elevated the release of PGE into the supernatants. Menhaden fish oil–AM stimulated with endotoxin released 70% less PGE than those from LRD piglets ($P < 0.05$). When we examined another source of immune cells (*i.e.*, resident peritoneal macrophages), similar trends were noted. However, these data were considerably more variable than the AM data. The only diet-related difference that approached statistical significance

TABLE 3

Mean Fatty Acid Content of Total Splenocyte, Alveolar Macrophage and Thymus Fatty Acids from Three-Week-Old Piglets Nursing Sows Fed Diets Containing 7% (by wt) Either Lard (LRD) or Menhaden Fish Oil (MFO)[a]

	Immune tissues/cell types								
	Splenocytes			Alveolar macrophages			Thymus		
	Maternal fat source								
Fatty acid[b]	LRD[c]	MFO		LRD	MFO		LRD	MFO	
	(g/100 g total fatty acids)								
14:0	0.5	0.6	0.1[d]	0.9	1.6	0.3[d]	1.0	1.4	0.2[d]
16:0	25.3	23.0	1.6	37.6	38.4	1.3	25.5	26.7	1.2
16:1	1.3	1.9	0.2	2.3	2.7	0.3	2.6	3.2	0.6
18:0	13.3	12.3	0.9	6.7	7.2	0.4	9.9	10.9	0.8
18:1n-9	14.3	11.8	1.1	13.7	10.2[e]	1.1	18.2	12.9[e]	1.5
18:1n-7	3.0	2.5	0.2	2.0	1.8	0.1	5.1	4.6	0.2
18:2n-6	7.4	8.7	0.8	5.3	4.8	0.4	10.3	9.2	0.6
20:3n-6	1.2	1.0	0.2	1.0	0.5[f]	0.1	1.5	1.6	0.2
20:4n-6	20.6	8.8[f]	1.5	15.9	5.7[f]	0.6	15.3	7.5[f]	0.9
20:5n-3	0.1	12.2[f]	1.0	0.2	8.7[f]	0.6	0.1	6.9[f]	0.8
22:4n-6	2.7	0.3[f]	0.2	3.4	0.4[f]	0.4	2.1	0.3[f]	0.1
22:5n-6	trace	trace		0.6	trace[f]	0.1	trace	trace	
22:5n-3	2.1	5.3[f]	0.2	2.9	6.5[f]	0.3	1.5	3.6[f]	0.2
22:6n-3	2.3	6.3[f]	0.3	2.2	6.6[f]	0.4	1.3	5.3[f]	0.4
Total SAT[g]	39.1	35.9	2.3	45.2	47.2	1.2	36.4	39.0	1.8
Total MONO[h]	18.6	16.2	1.0	18.0	14.7[e]	1.1	25.9	20.7[e]	1.6
Total n-6 PUFA[i]	32.4	19.0[e]	2.4	26.3	11.6[f]	1.0	30.3	19.2[f]	1.2
Total n-3 PUFA[j]	4.6	24.1[f]	1.3	5.3	22.1[f]	1.1	3.0	16.2[f]	1.4
Total PUFA[k]	37.5	43.4	2.9	31.9	33.8	1.4	32.6	35.0	2.1

[a]Sows were fed diets containing 70 g/kg LRD or MFO for one week prior to farrowing. Piglets nursed sows within the same treatment group. Samples were collected from piglets upon weaning (3 wk). Abbreviations as in Table 2.
[b]Fatty acids are denoted by the number of carbons: number of double bonds, followed by the position of the first double bond relative to the methyl-end (n-) of the fatty acid.
[c]Values are expressed as means (n = 6) for fatty acids that accounted for 0.7% or more of the total fatty acids present in the sample; trace, less than 0.3% of the total fatty acids.
[d]Pooled SEM.
[e]Mean MFO value significantly different from LRD value ($P < 0.05$).
[f]Mean MFO value significantly different from LRD value ($P < 0.0005$).
[g]Sum total area percentage of 14:0, 16:0 and 18:0.
[h]Sum total area percentage of 16:1, 18:1n-9, 18:1n-7 and 20:1.
[i]Sum total area percentage of 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6.
[j]Sum total area percentage of 18:3n-3, 18:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.
[k]Sum total area percentage of fatty acids listed in footnotes i and j.

TABLE 4

Prostaglandin E (PGE) Production by Alveolar Macrophages (AM) and Resident Peritoneal Cells from Piglets Suckling Sows Fed Diets Containing 7% (by wt) of Either Lard (LRD) or Menhaden Fish Oil (MFO)

	Immune cell types[a]			
	AM		Peritoneal cells	
	Maternal fat source			
Culture conditions[b]	LRD	MFO	LRD	MFO
	(pg/0.1 mL)[c]			
Basal	94[d]	8[e]	1511	777
A23187-stimulated	156	20	2902	1245
Endotoxin-stimulated	1490[d]	425[e]	2802	2764

[a]Resident peritoneal cells were collected from three to four week old piglets by filling the peritoneal cavity with ≈400 mL of sterile ice-cold Hank's, and AM were collected from excised lungs with two 60-mL flushes of ice-cold Hank's.
[b]The isolated immune cells (2.5×10^6/well) were incubated (37°C and 5% CO_2) 24-well cell culture plates. After 4 h, nonadherent cells were washed away and remaining cells were co-cultured with media alone for 24 h (basal), 2 μg/mL of calcium ionophore for 4 h (A23187-stimulated) or 5 μg/mL of lipopolysaccharide from *E. coli* O111:B4 for 24 h (endotoxin-stimulated).
[c]The concentration of PGE in the cell supernatants was determined by enzyme immunoassay (Advanced Magnetics, Inc.). Data were log transformed to achieve homogeneity of variance then analyzed by ANOVA. Values shown represent the mean (n = 4-6).
[d,e]Values within rows that do not share a common superscript are statistically different ($P < 0.05$).

($P < 0.08$) was with the A23187-stimulated peritoneal cells preparations.

The production of other eicosanoids by isolated pig AM was also influenced by supplementing the sow's diet with fish oil. In a follow-up experiment the impact of n-3 enrichment on LTB and TXB production by piglet AM was investigated. Cells were isolated from three to four week old piglets born to sows fed either fish oil- or lard-containing diets as described above. However, for this study AM were collected from piglets after weaning. In order to prevent the reversal of the maternal fat source effects, weaned piglets were fed diets which contained the same fat source assigned to the sow.

As in the first study, spontaneous and calcium ionophore-stimulated PGE production was significantly lower in the MFO piglets as compared with the LRD piglets (Fig. 1, top panel). Spontaneous thromboxane A production (measured as the stable metabolite, TXB) was significantly lower in the MFO piglets as compared with the LRD piglets (Fig. 1, middle panel). Ionophore stimulation greatly increased TXA release in both MFO and LRD piglets (6.5-fold *vs.* 3-fold, respectively). However, the net release by stimulated AM from the MFO piglets was still significantly lower than that from the LRD piglets ($P < 0.05$). The basal release of LTB was significantly reduced in the MFO pigs compared with LRD pigs (Fig. 1, bottom panel). As observed with other eicosanoids measured, LTB release was greatly enhanced by co-culturing the cells with the calcium ionophore, A23187. However, when AM were stimulated with calcium ionophore, release of LTB was similar for both MFO and LRD pigs.

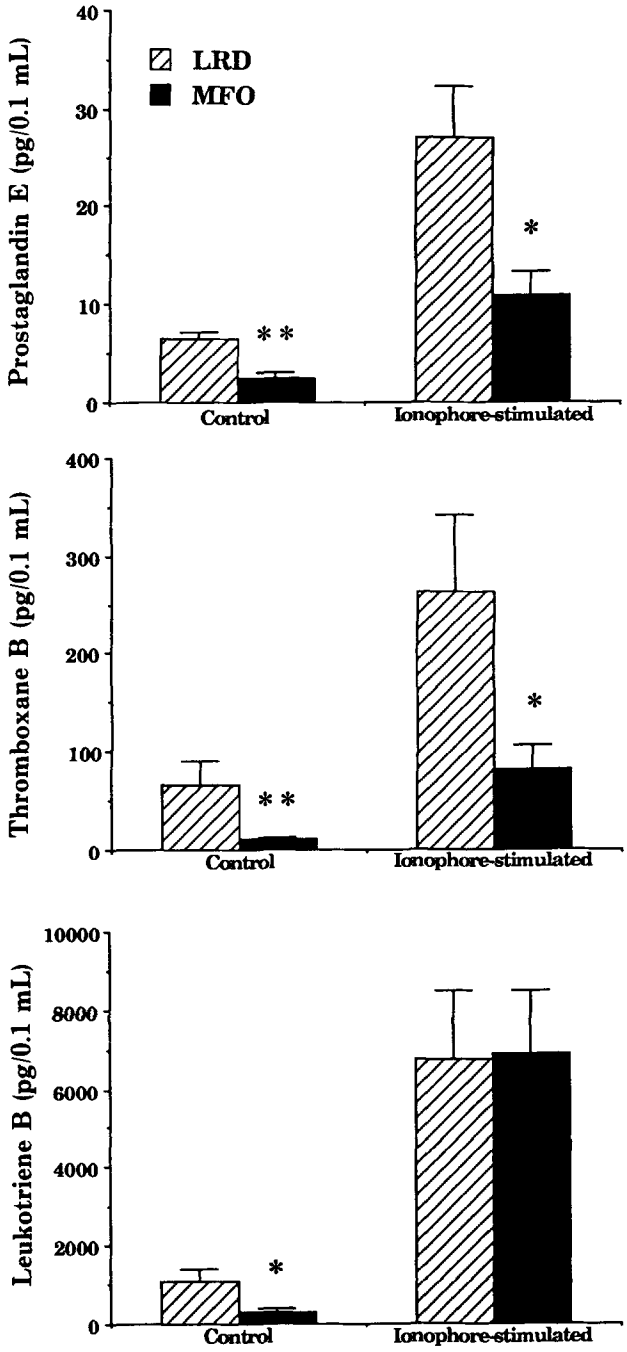

FIG. 1. Production and release of prostaglandin E (PGE), thromboxane B (TXB) and leukotriene B (LTB) from alveolar macrophages (AM) isolated from three- to four-week-old pigs suckling menhaden fish oil-fed (MFO) or lard-fed (LRD) sows. After adherence, 2.5 × 10^6 AM were rinsed and then incubated for 1 h in the presence of 2 μg/mL of calcium ionophore (A23187) or medium with vehicle only (controls). Supernatants were analyzed for PGE, TXB and LTB by enzyme immunoassay. Data represent the means (n = 6) with error bars representing the SEM. Values for the MFO group are significantly lower from the LRD group (*$P < 0.05$; **$P < 0.005$) as determined by ANOVA.

DISCUSSION

Our data demonstrate that sufficient n-3 PUFA were in the milk of fish oil-fed sows such that the immune tissues and cells from pigs suckling these sows were enriched with n-3 PUFA. The most substantial changes in immune cell fatty acid profiles occurred in the relative levels of the eicosanoid precursors, AA (20:4n-6) and EPA (20:5n-3). Immune cell AA levels dropped by one-half in the MFO pigs as compared to the LRD pigs. In the MFO pigs the level of EPA in the immune cells exceeded the AA that remained. This may seem somewhat surprising in view of the relatively small amount of EPA in the milk of fish oil-fed sows (≈3% of the total fatty acids in the milk; see Ref. 7). However, such a selective enrichment of EPA is probably a consequence of the greater rate of oxidation of the fatty acids <20 carbons in length and the preferential incorporation of 20–22 carbon fatty acids into membrane phospholipids. In 1991, Arbuckle *et al.* (10) reported that the fatty acid composition of a number of pig tissues (*e.g.*, brain, liver and red blood cells) could be enriched with n-3 PUFA by providing pigs a formula supplemented with menhaden fish oil. Enrichment of immune cells with n-3 fatty acids has been demonstrated in rats (11,12), mice (13), chickens (14) and humans (15). However, in each of these cases, the n-3 PUFA were provided directly in the diet of the experimental subject.

As predicted, the changes in immune cell fatty acid composition led to a significant alteration in eicosanoid production by these same cells. The reduction in prostaglandin production by immune cells enriched with n-3 PUFA has been documented in several other species, including rats (11,12), mice (16–18), chickens (19) and humans (15,20). This is the first report of porcine immune cell eicosanoid production being altered by enriching cellular n-3 PUFA content. n-3 PUFAs alter eicosanoid produc-

tion through metabolic competition between the two major families of PUFA, the n-6 and the n-3. Upon fish oil feeding, EPA and other n-3 PUFA displace some of the AA from immune cell membrane phospholipids. Because EPA is a poor substrate for the cyclooxygenase enzyme, its conversion into prostaglandins is limited. Therefore, when cellular fatty acid profiles are altered as described above, these changes lead to a reduced capacity for prostaglandin and thromboxane synthesis. Additionally, it has been reported that docosahexaenoic acid (22:6n-3) reduces *in vivo* macrophage eicosanoid production (21). Thus the decrease in AA and the increase in n-3 PUFA in the immune cells from the MFO-pigs probably account for most of the changes in eicosanoid production observed in our study.

The impact of n-3 PUFA enrichment on production of LTB is more complex than that described above for PGE and TXB. Unlike for the cyclooxygenase, 20:5n-3 is a good substrate for the 5-lipoxygenase enzyme. Thus one would expect the production of substantial amounts of LTB from this n-3 fatty acid when its levels are elevated. The findings of Leitch *et al.* (22) and Lee *et al.* (20) support such a conclusion. However, the form of eicosanoid analysis used in this study (*i.e.,* enzyme immunoassay) does not allow us to distinguish between LTB_4 (derived from AA) and LTB_5 (derived from EPA). It seems highly probable that a sizable proportion of the immunoreactive LTB measured in this study was LTB_5. It is unclear why we observed a difference between the LRD- and MFO-pigs in the basal release of LTB, but not upon ionophore-stimulation. It is possible that basal release of EPA from membrane phospholipids is less than AA or that reincorporation is faster. Alternatively, differential alteration of AA and EPA levels across distinct phospholipid pools may be important.

AA metabolites play an important role in the pathophysiologic responses of the lung, and AM are a major source for these products (23). The reduction in PGE and TXB production and the replacement of LTB_4 with LTB_5 could be of biological significance. LTB_5 has been reported to be 1/30 to 1/60 as potent as LTB_4 in eliciting neutrophil chemotaxis (22). Thus immune tissues enriched with 20:5n-3 would be expected to cause less pro-inflammatory activity than those not enriched. Furthermore, a decline in infectious disease resistance has also been reported for n-3 PUFA-enriched animals. D'Ambola *et al.* (24) reported that *in vivo* pulmonary bacterial clearance in neonatal rabbits was diminished by fish oil-supplementation. Feeding mice fish oil significantly reduced survival from a live bacterial challenge (25). Whether such changes in immune status are a consequence of altered eicosanoid production remains to be determined.

In conclusion, we believe these data support our hypothesis and that the transfer of n-3 PUFA from fish oil-fed sows to the nursing pig is of sufficient magnitude to elevate n-3 PUFA levels in immune cells, which leads to a significant alteration in eicosanoid production by these same cells. This may be of practical importance as a variety of immune and inflammatory processes have been shown to have been influenced by eicosanoids and n-3 PUFA. Therefore, we believe that further studies are warranted to examine the impact of maternal n-3 PUFA intake on the immunological and inflammatory responses in the newborn.

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The Hypotriglyceridemic Effect of Eicosapentaenoic Acid in Rats Is Reflected in Increased Mitochondrial Fatty Acid Oxidation Followed by Diminished Lipogenesis

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The effect of eicosapentaenoic acid (EPA) on fatty acid oxidation and on key enzymes of triglyceride metabolism and lipogenesis was investigated in the liver of rats. Repeated administration of EPA to normolipidemic rats resulted in a time-dependent decrease in plasma triglycerides, phospholipids and cholesterol. The triglyceride-lowering effect was observed after one day of feeding whereas lowering of plasma cholesterol and phospholipids was observed after five days of treatment. The triglyceride content of liver was reduced after two-day treatment. At that time, increased mitochondrial fatty acid oxidation occurred whereas mitochondrial and microsomal glycerophosphate acyltransferase was inhibited. The phosphatidate phosphohydrolase activity was unchanged. Adenosine triphosphatecitrate lyase, acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase were inhibited during the 15 d of EPA treatment whereas peroxisomal β-oxidation was increased. At one day of feeding, however, when the hypotriglyceridemic effect was established, the lipogenic enzyme activities were reduced to the same extent in palmitic acid-treated animals as in EPA-treated rats. In cultured rat hepatocytes, the oxidation of [^{14}C]palmitic acid to carbon dioxide and acid-soluble products was stimulated in the presence of EPA. These results suggest that the instant hypolipidemia in rats given EPA could be explained at least in part by a sudden increase in mitochondrial fatty acid oxidation, thereby reducing the availability of fatty acids for lipid synthesis in the liver for export, *e.g.*, in the form of very low density lipoproteins, even before EPA induced peroxisomal fatty acid oxidation, reduced triglyceride biosynthesis and diminished lipogenesis.
Lipids 28, 683–690 (1993).

Marine oils are rich in polyunsaturated fatty acids of the n-3 family, especially eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Dietary intake of these fatty acids reduces plasma triglycerides and inhibits production of very low density lipoproteins (VLDL) in human hyper- and normolipidemic subjects and in experimental animals (1–12). The mechanism of this effect has also been studied in cultured cells (4,5) and the results obtained indicate that inhibited lipogenesis (13) and decreased triglyceride synthesis are important factors that contribute to the reduced secretion of VLDL triglycerides.

Glycerol 3-phosphate acyltransferase which catalyzes the initial esterification step in triglyceride synthesis, could not be shown to be involved in the observed triglyceride-lowering effect of fish oil (5,14). On the other hand, contradictory results were obtained in regard to the potential role of phosphatidate phosphohydrolase. Wong and Marsh (15), Marsh *et al.* (16) and Al-Shurbaji *et al.* (14) reported a decrease in phosphatidate phosphohydrolase activity in response to fish oil while in other studies such an inhibition could not be demonstrated (17,18). Differing results on the effect of ω3 fatty acids on diacylglycerol acyltransferase activity were also reported (14, 16–19).

We have recently demonstrated that EPA (95% pure) shows both hypotriglyceridemic and hypocholesterolemic properties (18,20). Our data suggested that the hypotriglyceridemic effect may have been due in part to a reduced supply of fatty acids for hepatic triglyceride synthesis because of increased fatty acid oxidation. The present investigation was undertaken to further explore how EPA feeding affects hepatic lipid metabolism, especially in mitochondria, and how it affects several of the enzymes involved in triglyceride biosynthesis and lipogenesis.

MATERIALS AND METHODS

Chemicals. Ethyl EPA, purity 95%, was obtained from Norsk Hydro AS, Research Center (Porsgrunn, Norway). Palmitic acid (PMA) was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Møllegaard Breeding Laboratory (Ejby, Denmark), weighing 182–210 g, were housed in groups of three in grid-bottom metal wire cages in a room maintained at a 12-h light/dark cycle and at a temperature of 20 ± 3°C. The animals were acclimated for at least 1 wk before the start of the experiment. All animals had free access to food and water and were given a standard rat diet (18). EPA and PMA were suspended in 0.5% sodium carboxymethylcellulose (CMS) and 0.5% tocopherol.

In the time study a daily dose of 1500 mg/kg body weight of EPA or PMA was administered by gastric intubation in volumes of 0.75–1.9 mL. The EPA-treated animals were killed on day 1, 3, 5, 10 or 15 after 12 h of fasting while animals treated with PMA were killed on day 2 or 10 and served as control groups. The animal experiments were approved by the local Ethics Committee for Animal Experiments.

Body weights were recorded daily. At the end of the experiments, the rats were fasted and weighed. Cardiac puncture was performed under neuroleptic anesthesia to

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Abbreviations: ADGAT, acyl-CoA:diacylglycerol acyltransferase; ATP, adenosine triphosphate; CMS, carboxymethylcellulose; CPCT, CTP:phosphocholine cytidylyltransferase; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); L-fraction, peroxisome-enriched fraction; M-fraction, mitochondrial fraction; NADH, nicotinamide adenine dinucleotide; P-fraction, microsomal fraction; PMA, palmitic acid; S-fraction, cytosolic fraction; VLDL, very low density lipoprotein.

obtain blood samples, and the liver was removed immediately, weighed and then chilled on ice. Plasma was prepared by centrifugation of the clotted whole blood at 1000 × *g* for 10 min at 5°C.

Preparation of cultured hepatocytes. Hepatocytes were prepared according to Seglen (21). In short, the rats were anesthetized with barbiturates, and hepatocytes were isolated by collagenase perfusion, washed and plated in Dulbecco's modified Eagle's medium containing *N*-(2-hydroxyethyl)pyperazine-*N*-(2 ethanesulfonic acid (Hepes) (20 mM), Ultroser G (2%) and gentamicin (50 μL) at a density of 3×10^6 cells/dish or culture flask (1×10^6 cells/mL). The culture medium was replaced after 6–7 h (2 mL/dish, medium as before). The cells were incubated at 37°C in an atmosphere of air containing 5% CO_2.

Fatty acid oxidation. Fatty acid oxidation to acid soluble products and CO_2 (to determine the rate of β-oxidation) was measured as described by Christiansen *et al.* (22). To the cultured hepatocytes was added 2 mL Dulbecco's modified Eagle's medium containing Hepes (20 mM), gentamicin, [1-^{14}C]PMA (0.5 μCi/mL, 200 μM) and 500 μM L-carnitine hydrochloride, and the hepatocytes were incubated for 9 h in the presence of EPA.

The culture flasks contained a center well (Kontes, Vineland, NJ) and a folded filter paper and were closed with stopper tops (Kontes). After incubation, 300 μL of phenylethylamine/methanol (1:1, vol/vol) was added to the center well and 300 μL of $HClO_4$ (1 M) to the cells through the stopper top by means of syringe, and the flasks were further incubated for 1 h at room temperature to trap all $^{14}CO_2$. After incubation, the well was cut off and radioactivity was measured by liquid scintillation counting.

Analytical methods. Individual livers were homogenized in ice-cold sucrose medium [0.25 M sucrose in 10 mL Hepes buffer, pH 7.4 and 1 mM ethylenediaminetetraacetic acid (EDTA)] using a Potter-Elvehjem homogenizer at 720 rpm and two strokes of a loose-fitting Teflon pestle. After centrifugation, the resulting nuclear plus postnuclear fractions were used as the total homogenate.

A mitochondrial-enriched fraction (M-fraction) was prepared from the postnuclear fraction at 12000 × *g* for 10 min (SS 34 rotor). A peroxisome-enriched fraction (L-fraction) was prepared by centrifugation at 14000 × *g* for 30 min. A microsomal-enriched fraction (P-fraction) was isolated from the postperoxisomal fraction at 100000 × *g* for 1 h (Ti 60 rotor). The remaining supernatant was collected as the cytosolic fraction (S-fraction).

Protein was assayed using the Bio-Rad protein assay kit (BioRad, Richmond, CA).

Lipid analyses were carried out using the monotest cholesterol enzymatic kit (Boehringer Mannheim, Germany) and the Biopak triglyceride enzymatic kit (Biotrol, Paris, France).

Plasma free fatty acids were determined by an enzymatic colorimetric method (WACO Nefa C) (23).

Enzyme assays. Enzymatic activities of palmitoyl-CoA dependent dehydrogenase measuring peroxisomal β-oxidation with palmitoyl-CoA as substrate (20,24), fatty acyl-CoA oxidase (20), carnitine palmitoyl-transferase with no malonyl-CoA present (20,24), palmitoyl-CoA synthetase (21), palmitoyl-CoA hydrolase (24), phosphatidate phosphohydrolase (25), CPCT (CTP:phosphocholine cytidylyltransferase) (25), fatty acid synthetase (25), mitochondrial β-oxidation (25), glucose-6-phosphate dehydrogenase (25) and diacylglycerol acyltransferase (26) were determined as described earlier.

Adenosine triphosphate (ATP)-citrate lyase activity was assayed according to Rose-Kahn and Bar-Tana (27). The reaction mixture contained 100 mM Tris-HCl buffer, pH 8.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.33 mM CoA, 0.14 mM nicotinamide adenine dinucleotide (NADH), two units of malate dehydrogenase, K-citrate as stated and 5–10 μL of freshly thawed supernatant as enzyme source in a final volume of 1 mL. After 5 min of preincubation of the enzyme with the reaction mixture, the reaction was started by the addition of 50 μL of 0.1 M ATP and the oxidation of NADH recorded at 340 nm at room temperature. Activity was expressed in nanomoles of citrate converted to acetyl-CoA per min.

Acetyl-CoA carboxylase was determined in the cytosolic fraction as incorporation of $NaH^{14}CO_3$ into malonyl-CoA (28).

Statistical analysis. Unless otherwise stated, the *in vivo* results are means ± SD of duplicate measurements on three animals of each experimental group and of six control animals. The effects of PMA and EPA treatment were tested by a two-way analysis of variance. $P < 0.05$ was used as the significance levels to express PMA or EPA effects. For isolation of some cellular fractions from livers, the postnuclear fractions from three animals were pooled. In this case the results are given only as means. Results from cultured rat hepatocytes are presented as mean ± SD of at least three culture dishes/flasks of hepatocytes from two or more animals. Statistical analysis was by Student's *t*-test and $P < 0.05$ was taken to be statistically significant.

RESULTS

Body and liver weights. The rats fed a normolipidemic diet supplemented with EPA and PMA gained weight per day at the same rate as did the controls. Food consumption was similar in each experimental group irrespective of the dietary regimen indicating that appetite was not affected and that the acids were well tolerated (data not shown). All animals treated with the fatty acids as a function of time appeared healthy and looked and behaved normal. No hepatomegaly resulted after PMA or EPA feeding even at a dose of 1500 mg/day/kg body weight (Table 1). The hepatic concentration of protein remained unchanged in all experimental groups (Table 1).

Plasma and liver lipids. Repeated administration of EPA to rats caused a significant ($P < 0.05$) time-related reduction of plasma triglycerides (50–60% decrease, Fig. 1A), cholesterol (30–50% decrease, Fig. 1A) and phospholipids (30–50% decrease, Fig. 1A). The triglyceride-lowering effect was significant ($P < 0.05$) after one day of treatment, whereas significant ($P < 0.05$) lowering of plasma cholesterol and phospholipids was observed after 5 d of EPA treatment. Consistent with previous observations (20), plasma triglycerides, cholesterol and phospholipids were not significantly lowered after administration of PMA (Fig. 1B).

Figure 1A shows that treatment of rats with EPA tended to decrease the plasma free fatty acids (Fig. 1A). The reduction was not significant. However, PMA feeding increased plasma free fatty acids (Fig. 1B).

TABLE 1

Time-Dependent Changes of Liver Weight and Liver Lipids in Rats Treated with EPA or PMA at a Dose of 1500 mg/d/kg Body Weight[a]

Parameters	Compound	Exposure time (days) 0	1	2	3	5	10	15
Liver g/g	PMA	3.32 ± 0.21		3.18 ± 0.35			3.19 ± 0.18	
body weight	EPA	3.23 ± 0.32	3.39 ± 0.15	4.14 ± 0.21	3.29 ± 0.26	3.25 ± 0.27	3.52 ± 0.20	3.55 ± 0.21
Protein	PMA	176.6 ± 10.2		168.7 ± 3.8			153.0 ± 10.7	
mg/g liver	EPA	178.2 ± 10.2	170.7 ± 5.4	179.0 ± 7.0	161.3 ± 3.7	177.2 ± 5.9	169.1 ± 6.3	168.3 ± 6.5
Triglycerides	PMA	7.88 ± 1.60		7.58 ± 1.20			7.89 ± 1.50	
μmol/g liver	EPA	7.89 ± 1.50	6.14 ± 1.66	4.36[b] ± 1.62	4.72[b] ± 0.65	4.60[b] ± 2.05	4.66[b] ± 0.92	5.74 ± 1.47
Phospholipids	PMA	18.7 ± 3.8		19.4 ± 1.3			15.7 ± 3.4	
μmol/g liver	EPA	19.1 ± 3.6	19.5 ± 2.7	16.9 ± 0.3	22.5 ± 1.8	17.9 ± 1.6	21.6 ± 0.9	20.8 ± 1.6
Cholesterol	PMA	3.2 ± 0.5		2.4 ± 0.8			3.0 ± 0.5	
μmol/g liver	EPA	3.1 ± 0.5	2.9 ± 0.6	2.2 ± 0.2	4.1 ± 0.5	4.5 ± 1.5	4.1 ± 1.4	3.6 ± 0.5

[a]The hepatic values represent means ± SD of six control animals and groups of three rats in each experimental group. Abbreviations: EPA, eicosapentaenoic acid; PMA, palmitic acid.
[b]$P < 0.05$ for difference between control and treated rats.

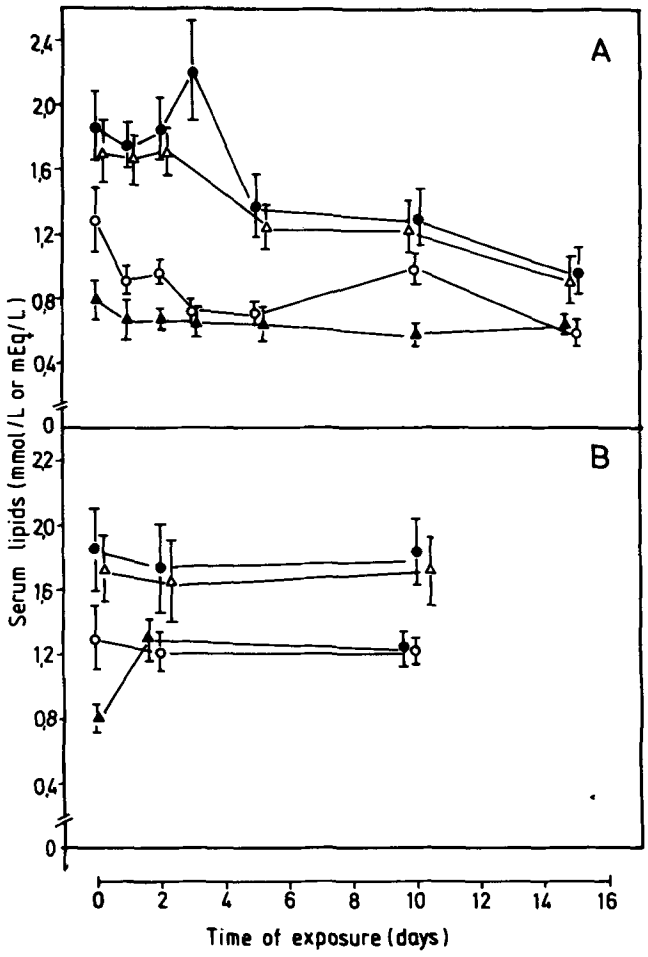

FIG. 1. Time course of plasma triglycerides (○), cholesterol (△), phospholipids (●) and free fatty acids (▲) in rats given eicosapentaenoic acid (A) or palmitic acid (B). The values represent means ± SD of nine control animals and three rats in each experimental group. Plasma triglycerides, cholesterol and phospholipids are given in mmol/L, whereas plasma free fatty acids are in Eq/L.

EPA caused a significant ($P < 0.05$) reduction of the hepatic concentration of triglycerides (Table 1) during the first two days of treatment when compared to 0 d. Repeated administration of EPA or PMA had no effect on the concentration of hepatic cholesterol and phospholipids (Table 1).

Mitochondrial and peroxisomal β-oxidation. Figure 2A shows that in animals fed EPA the mitochondrial oxidation of fatty acids increases. This was seen with palmitoyl-CoA and especially with palmitoyl-L-carnitine as substrate where oxidation of palmitoyl-L-carnitine was rapidly increased to its maximum after one day of feeding EPA. At that time, mitochondrial β-oxidation was increased 1.8-fold. The oxidation of palmitoyl-CoA was maximally increased after 2 d of feeding. Subsequently, in EPA-treated rats, the oxidation of palmitoyl-CoA and palmitoyl-L-carnitine fell to almost normal values (15 d).

Repeated administration of EPA tended to increase carnitine palmitoyltransferase activity in a time-dependent manner (Table 2). At 15 d of feeding, significant enzyme activity was observed.

EPA treatment also caused an increase in both fatty acyl-CoA oxidase (Fig. 2B) and peroxisomal β-oxidation (Table 2) after 5–15 d of feeding. The two enzyme activities were increased about 3- and 2.5-fold, respectively, after 15 d of feeding. Peroxisomal β-oxidation was not affected up to 3 d of EPA feeding (Table 2), whereas fatty acyl-CoA oxidase showed a significant increase in that feeding period (Fig. 2B).

Effect of EPA on the oxidation of [^{14}C]PMA in cultured hepatocytes. The oxidation of [^{14}C]PMA to carbon dioxide and acid-soluble products was stimulated in cultured hepatocytes in the presence of EPA (Fig. 3). This effect was already seen at the lowest concentration of EPA used (50 μM).

Palmitoyl-CoA synthetase and palmitoyl-CoA hydrolase. The level of long-chain acyl-CoA is likely to reflect the balance between the rate of activation of fatty acids to acyl-CoA and the rate of utilization for oxidation, esterification and hydrolysis. As adaptive changes in the

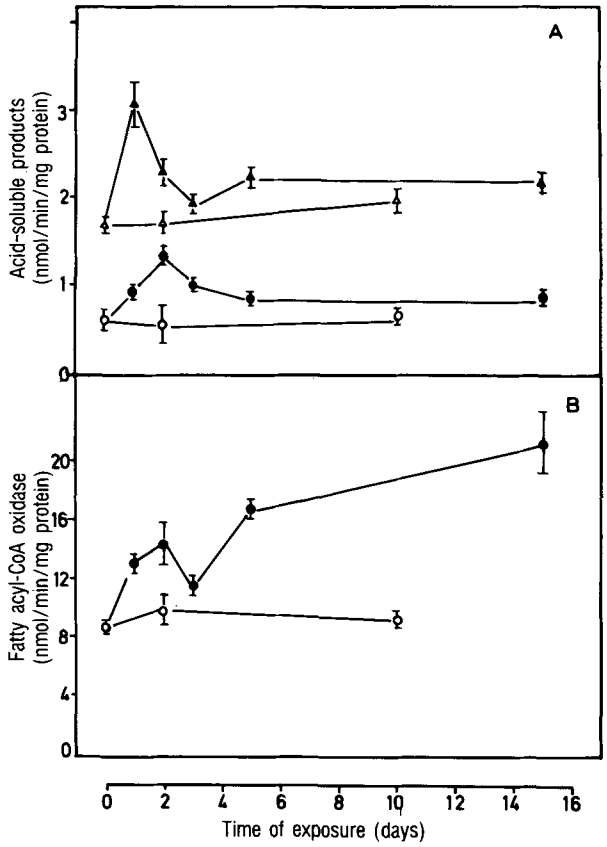

FIG. 2. Time course of β-oxidation of [1-^{14}C]palmitoyl-L-carnitine (△,▲) and [1-^{14}C]palmitoyl-CoA (○,●) (A) and fatty acyl-CoA oxidase activity (○,●) (B) in liver homogenates of rats treated with eicosapentaenoic acid (▲,●) or palmitic acid (△,○). The values represent means ± SD of six control rats and three animals in each experimental group.

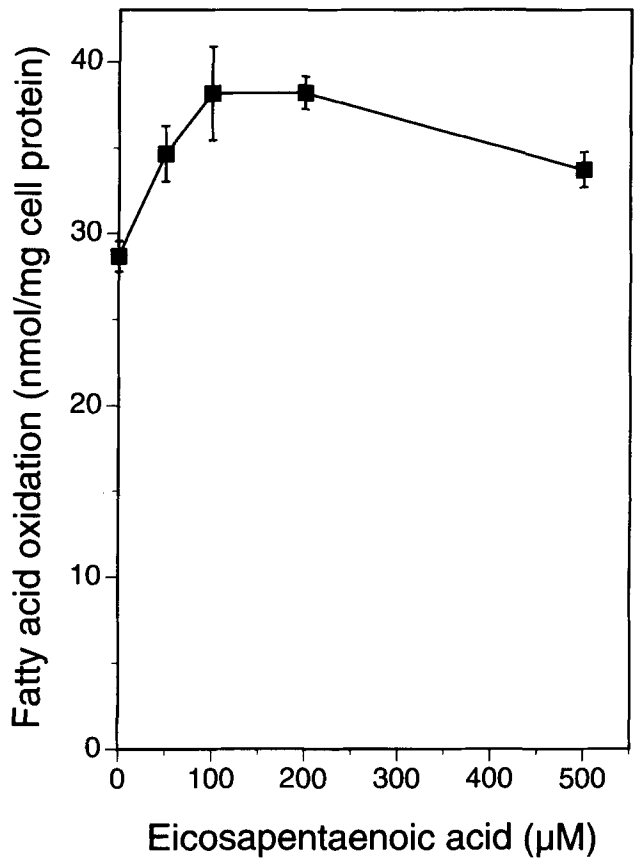

FIG. 3. Effect of eicosapentaenoic acid on fatty acid oxidation of [^{14}C]palmitic acid by cultured rat hepatocytes. Hepatocytes were incubated for 4 h in Dulbecco's modified Eagle's medium containing Hepes (20 mM), L-carnitine hydrochloride (0.5 mM) and [1-^{14}C]palmitic acid (0.25 μCi/mL, 200 μM) and various concentrations of eicosapentaenoic acid. After incubation, labelled carbon dioxide and acid soluble radioactivity were collected as described in Materials and Methods. Oxidation of palmitic acid in the presence of oleic acid (200 μM) was 33.3 ± 1.2 nmol/mg cell protein. Data are presented as means ± SD of triplicate cultures and show total fatty acid oxidized (carbon dioxide plus acid soluble radioactivity).

activities of the enzymes involved in these pathways may reflect alterations in the metabolic flux through the pathways, we examined whether palmitoyl-CoA synthetase activity and palmitoyl-CoA hydrolase activity were changed upon EPA feeding.

Figure 4A shows that administration of EPA significantly ($P < 0.05$) increased palmitoyl-CoA synthetase activity in total liver homogenates at 10 d of feeding. This increase may be attributed to stimulation of peroxisomal palmitoyl-CoA synthetase activity (Fig. 4A).

Repeated administration of EPA to rats had no effect on palmitoyl-CoA hydrolase activity in total liver homogenates, although there may have been a small effect in microsomes (Fig. 4B). In contrast to feeding sulfur-substituted fatty acids, which increase the cytosolic palmitoyl-CoA hydrolase activity (20), EPA-treatment tended to decrease this activity (Fig. 4B).

Glycerophosphate acyltransferase and phosphatidate phosphohydrolase. Upon EPA feeding, both the mitochondrial and microsomal glycerophosphate acyltransferase activities were decreased within 2 d (Table 3). Subsequently, the activities rose almost to normal values (3 d), and showed stimulation at 15 d. At this time, the microsomal glycerophosphate acyltransferase activity was increased 1.5-fold whereas the mitochondrial enzyme activity showed a nearly 1.3-fold increase (Table 3).

Repeated administration of EPA had no effect on the phosphatidate phosphohydrolase activity associated with the microsomal and cytosolic fractions (Table 3).

TABLE 2

Time Course of Carnitine Palmitoyltransferase of Rats Treated with Eicosapentaenoic Acid[a]

Time of exposure (days)	Carnitine palmitoyltransferase (nmol/min/mg protein)		Peroxisomal β-oxidation (nmol/min/mg protein)
	Total liver homogenates	Mitochondrial fraction	
0	8.37 ± 1.42	26.57 ± 2.82	3.03 ± 0.32
1	8.76 ± 0.13	28.14	4.04 ± 0.82
2	9.17 ± 0.53	28.05	4.40 ± 0.50
3	9.65 ± 0.91	32.24	3.68 ± 0.55
5	10.60 ± 0.79	31.84	5.91 ± 0.20[b]
15	11.89 ± 0.98[b]	35.97	6.92 ± 0.35[b]

[a]Data represent the means ± SD of nine control animals, means ± SD of three treated rats and means of three rats in each experimental group.
[b]$P < 0.05$ for differences between control and treated rats.

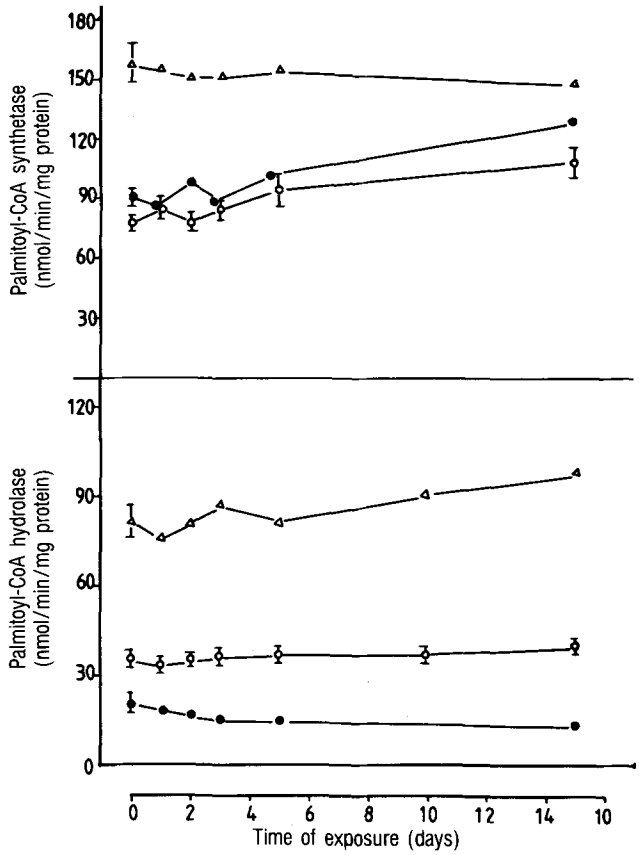

FIG. 4. Effect of eicosapentaenoic acid exposure on palmitoyl-CoA synthetase activity (top) and palmitoyl-CoA hydrolase activity (bottom). Palmitoyl-CoA synthetase activity in total liver homogenates (○), peroxisome-enriched fraction (●) and microsomal fraction (△). Palmitoyl-CoA hydrolase activity in total liver homogenates (○), microsomal fraction (△) and cytosolic fraction (●). Values for liver homogenates are reported as means ± SD for three to six rats. For the other cellular fractions values are reported as means of three rats in each experimental group.

Acyl-CoA:diacylglycerol acyltransferase (ADGAT) and CPCT. Hepatic ADGAT activity was increased with increasing feeding time of EPA. At 15 d, a 1.5-fold increase was observed (Table 4). The ADGAT activity was unchanged upon PMA administration (Table 4).

CPCT activity in the microsomal fraction (Table 4) and in the cytosolic fraction (data not shown) was unaffected by EPA and PMA administration.

Liver lipogenic enzymes. As inhibition of *de novo* fatty acid synthesis could account for a reduction of VLDL triglyceride secretion by the liver, we also investigated how EPA alters the activities of some lipogenic enzymes. Figure 5 and Figure 6 show that PMA and EPA inhibited the activities of ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthetase. However, the inhibition was most pronounced in the EPA-treated rats. At 2 d of feeding EPA, the activities of ATP-citrate lyase (Fig. 5) and acetyl-CoA carboxylase (Fig. 6A) were reduced by 45–50%. At this time, PMA decreased these enzyme activities by only about 15%. It is also noteworthy that EPA treatment reduced fatty acid synthetase activity after one day (Fig. 6B). Feeding EPA for 2 d reduced glucose-6-phosphate dehydrogenase activity to a greater extent than in PMA-treated rats (Table 5).

DISCUSSION

The studies show that a high intake of EPA lowers plasma triglyceride levels in rats (Fig. 1). This decrease is already established within 1 to 2 d of treatment (Fig. 1). Repeated administration of EPA also decreases the hepatic triglyceride content (Table 1). It seems likely, therefore, that the observed triglyceride lowering effect is due to a reduction in triglyceride biosynthesis.

Dietary fat and marine oils in particular increase fatty acid oxidation (29,30). The present study shows that pure EPA stimulates fatty acyl-CoA oxidase (Fig. 2) activity and peroxisomal β-oxidation (Table 2). It is therefore likely that induction of peroxisomal β-oxidation by high fat diets and fish oils is due to its content of EPA and DHA, *i.e.*, fatty acids which are poorly oxidized by mitochondria. The present study also shows that administration of pure EPA stimulates fatty acid oxidation in mitochondria. It is noteworthy that stimulation of mitochondrial β-oxidation is a rapid event, *i.e.*, it occurs after 1 and 2 d of feeding (Fig. 2) even before EPA induces peroxisomal β-oxidation. This mechanism has now been further evaluated using cells in culture. The present study has shown for the first time that mitochondrial β-oxidation is stimulated in

TABLE 3

Effect of Eicosapentaenoic Acid on the Activities of Glycerophosphate Acyltransferase (GPAT) and Phosphatidate Phosphohydrolase (PAP) in Liver of Rats[a]

Time of exposure	GPAT (nmol/min/mg protein)		PAP (nmol/min/mg protein)	
	Mitochondria	Microsomes	Microsomes	Cytosol
0	0.93 ± 0.10	1.56 ± 0.10	9.02 ± 0.26	4.43 ± 0.73
1	0.62 ± 0.12[b]	1.05 ± 0.15[b]	9.65 ± 0.20	4.47 ± 0.30
2	0.75 ± 0.05[b]	1.25 ± 0.05[b]	10.07 ± 0.15	4.43 ± 0.25
3	0.92 ± 0.15	1.52 ± 0.08	n.d.[c]	4.84 ± 0.50
5	0.99 ± 0.12	1.48 ± 0.20	8.67 ± 0.10	4.33 ± 0.35
10	1.05 ± 0.08	1.54 ± 0.10	8.80 ± 0.15	4.78 ± 0.28
15	1.15 ± 0.05[b]	2.38 ± 0.15[b]	9.86 ± 0.25	4.17 ± 0.35

[a]The values represent means ± SD of six control animals and groups of three rats in each experimental group.
[b]$P < 0.05$ for difference between control and treated rats.
[c]n.d., Not determined.

TABLE 4

Effect of Eicosapentaenoic Acid (EPA) and Palmitic Acid (PMA) on Acyl-CoA:Diacylglycerol Acyltransferase Activity (ADGAT) and CTP:Phosphocholine Cytidylyltransferase Activity (CPCT) in the Microsomal Fraction of Liver in Rats[a]

Days of treatment	Compound	ADGAT (nmol/min/mg protein)	CPCT (nmol/min/mg protein)
0		7.04 ± 1.50	0.25 ± 0.15
1	EPA	8.15 ± 0.60	0.18 ± 0.06
2	EPA	9.15 ± 0.20[b]	0.33 ± 0.04
3	EPA	9.85 ± 0.35[b]	0.26 ± 0.04
5	EPA	8.21 ± 1.05	0.28 ± 0.03
15	EPA	11.94 ± 0.30[b]	0.30 ± 0.04
2	PMA	7.39 ± 0.50	0.18 ± 0.07
10	PMA	7.53 ± 0.20	0.25 ± 0.06

[a]The tabulated values are the means ± SD of six control animals and means ± SD of three rats in each experimental group.
[b]$P < 0.05$ between the control and treated animals.

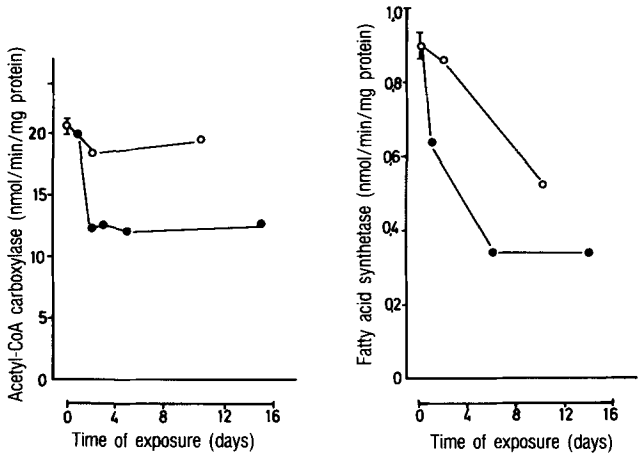

FIG. 6. Time course of acetyl-CoA carboxylase activity (left) and fatty acid synthetase activity (right) in cytosolic fractions of rats treated with eicosapentaenoic acid (●) or palmitic acid (○). The results are expressed as stated in the legend to Figure 1.

cultured hepatocytes in the presence of EPA (Fig. 3). The effects are in line with our results from fish oil feeding experiments where mitochondrial β-oxidation preceded peroxisomal β-oxidation (29). However, the data are in contrast to the findings of Neat *et al.* (31) that partially hydrogenated fish oil stimulated mitochondrial and peroxisomal oxidative activity simultaneously. In accordance with our results, Wong *et al.* (6) found that rats fed fish oil had increased ketone production.

As EPA rapidly increased mitochondrial fatty acid oxidation (Figs. 2 and 3) and decreased glycerol-3-phosphate acyltransferase activity within 1 d (Table 3), it is possible that the initial triglyceride-lowering effect (Fig. 1) of EPA is associated with reduced fatty acid availability for triglyceride biosynthesis through increased mitochondrial fatty acid oxidation, even before EPA induces peroxisomal β-oxidation (Table 2). Thus, the induction of peroxisomal β-oxidation is not a prerequisite for hypotriglyceridemia due to EPA. It is of interest that non-β-oxidizable thia fatty acids as well as EPA stimulated mitohondrial fatty acid oxidation before peroxisomal β-oxidation when fed to rats, thereby decreasing the flux of fatty acids into triglyceride formation and secretion (32). Thus, after feeding relatively poor substrates for mitochondrial β-oxida-

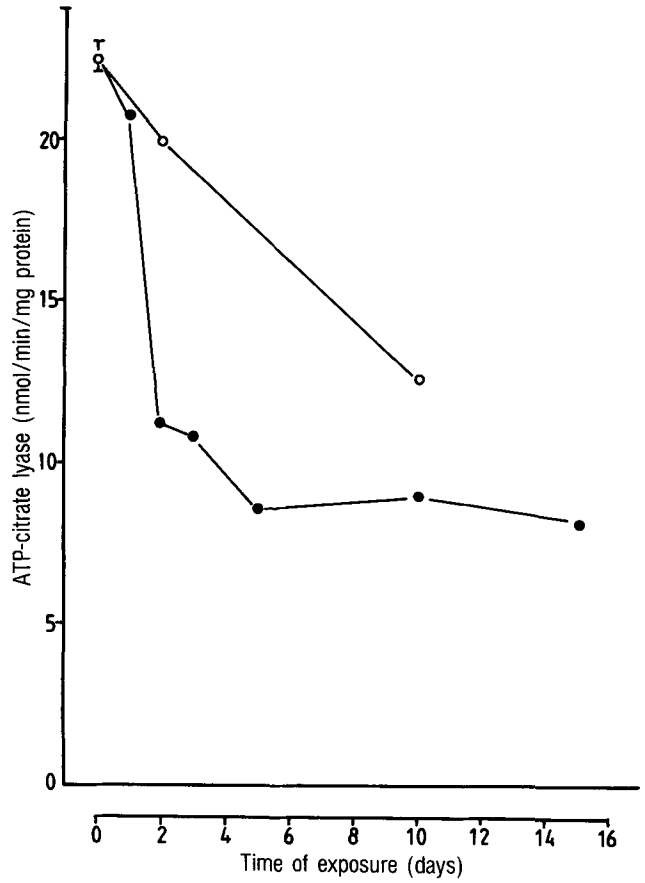

FIG. 5. Time course of adenosine triphosphate (ATP)-citrate lyase activity in cytosolic fractions of rats treated with eicosapentaenoic acid (●) or palmitic acid (○). Values are reported as means ± SD for nine rats and means of three rats in each experimental group.

TABLE 5

Effect of Eicosapentaenoic Acid (EPA) and Palmitic Acid (PMA) on the Activity of Glucose-6-Phosphate Dehydrogenase in the Liver Cytosolic Fraction of Rats[a]

Days of treatment	Compound	Glucose-6-phosphate dehydrogenase (% of control)
0		100
1	EPA	130 ± 20
2	EPA	70 ± 15
3	EPA	78 ± 10
5	EPA	44 ± 16
10	EPA	60 ± 10
15	EPA	38 ± 12
2	PMA	90 ± 10
10	PMA	61 ± 8

[a]The enzyme activity is calculated relative to that of pellet-fed controls (= 100%). The values represent means ± SD of three to six rats in each experimental group.

tion, the hypotriglyceridemic effect may be dissociated from induction of peroxisomal β-oxidation (33).

Glycerol-3-phosphate acyltransferase which catalyzes the first esterification step in triglyceride biosynthesis could be a potential site of regulation as both the mitochondrial and the microsomal glycerol-3-phosphate acyltransferase activities were reduced in EPA-treated rats after 1 d of feeding (Table 3). This is, however, in contrast to the findings of Wong *et al.* (5) and Al-Shurbaji *et al.* (14) where this enzyme was not found to be affected by ω3 fatty acids. The glycerol-3-phosphate acyltransferase activity, however, does not seem to correlate with the rate of triglyceride biosynthesis in EPA-treated rats. For longer feeding times, hepatic triglyceride concentrations were decreased (Table 1) whereas glycerol-3-phosphate acyltransferase activity was increased (Table 3).

Both phosphatidate phosphohydrolase and CPCT are found in the microsomal and in soluble fractions of liver homogenates, and the relative partitioning between cytosol and microsomes has been implicated in the regulation of glycerolipid synthesis (34–39). Phosphatidate phosphohydrolase is a key enzyme in the regulation of triglyceride biosynthesis, and we have recently reported that this enzyme was inhibited in tetradecylthioacetic acid-treated rates (25); however, no translocation of phosphohydrolase was observed. CPCT, the rate-limiting enzyme in the CDP-choline pathway, was stimulated by 3-thia fatty acids (25); however, no translocation of cytidylyltransferase was observed. The present results show that feeding pure EPA did not affect the phosphohydrolase and cytidylyltransferase activities in cytosolic as well as in microsomal fractions (Table 3). This observation is in accordance with Rustan *et al.* (17), but in contrast to the findings by Marsh *et al.* (16), Halminski *et al.* (34) and Al-Shurbaji *et al.* (14); these authors suggested that part of the triglyceride-lowering effect of ω3 fatty acids might be mediated by an inhibition of the phosphatidate phosphohydrolase.

The final step in the triglyceride synthetic pathway involves the conversion of 1,2-diglyceride to triglyceride which is catalyzed by ADGAT. ADGAT activity was increased in EPA-treated rats (Table 4) in accordance with data reported by Al-Shurbaji *et al.* (14), Halminski *et al.* (34), and Marsh *et al.* (16). As the activity of ADGAT does not seem to change in parallel with the rate of triglyceride biosynthesis, this step does not appear to be the site where EPA affects triglycerde levels, although this is not in agreement with the findings by Rustan *et al.* (17,19).

Although triglyceride formation seems to be reduced by EPA due to increased mitochondrial fatty acid oxidation, diminished lipogenesis may be a contributing factor to the overall lipid-lowering effect of EPA. Repeated administration of EPA inhibited several lipogenic enzyme activities, *i.e.,* ATP-citrate lyase (Fig. 5), acetyl-CoA carboxylase and fatty acid synthetase (Fig. 6), when compared with PMA-treated rats.

Acetyl-CoA carboxylase appears to be the rate-limiting enzyme in *de novo* fatty acid synthesis, and it is regulated by long-chain acyl-CoA (40). Long-chain acyl-CoA levels are rapidly increased after repeated administration of ω3 fatty acid and high fat diets (41). Figure 4 shows that the enzyme activity involved in the formation of long-chain acyl-CoA, *i.e.,* palmitoyl-CoA synthetase, was increased. Thus, the inhibitory effect of acetyl-CoA carboxylase activity in EPA-treated rats may be due to accumulation of long-chain acyl-CoA. Inhibition of acetyl-CoA carboxylase activity and glucose-6-phosphate dehydrogenase activity may reduce the production of malonyl-CoA and thereby reduce fatty acid synthetase activity. It is worth noting that at day 1 of feeding, when the hypotriglyceridemic effect of EPA was already established (Fig. 1), the lipogenic enzyme activities were reduced to the same extent in PMA-treated animals as in EPA-treated rats except for the fatty acid synthetase activity (Fig. 6B) which was more inhibited after EPA feeding. Thus, the inhibition of the *de novo* fatty acid synthesis could be secondary to increased fatty acid oxidation and thereby a reduced triglyceride formation.

Acyl-CoA esters accumulate in the liver of rats fed partially hydrogenated fish oil diets (41). Whether an increase in fatty acid oxidation represents an effort to minimize this pool should be considered. EPA is incorporated into triglycerides to a lesser degree than oleic acids (4,19). Thus, the presence of CoA-ester of EPA may result in an increased flux of fatty acids through both mitochondrial and peroxisomal fatty acid oxidation pathways.

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Metabolic Behavior in Rats of a Nonprotein Microemulsion Resembling Low-Density Lipoprotein

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A protein-free microemulsion (LDE) with a lipid composition resembling that of low-density lipoprotein (LDL) was used in metabolic studies in rats to compare LDE with the native lipoprotein. LDE labeled with radioactive lipids was injected into the bloodstream of male Wistar rats, and plasma kinetics of the labeled lipids were followed on plasma samples collected at regular intervals for 12 h after injection. The 24-h LDE uptake by different tissues was also measured in tissue samples excised after the animals had been sacrificed. We found that LDE plasma kinetics were similar to those described for native LDL [fractional clearance rate (FCR) of cholesteryl ester, 0.42 ± 0.11 h^{-1}]. The major site for LDE uptake was the liver, and the tissue distribution of the LDE injected radioactivity was as one would expect for LDL. To test whether LDE was taken up by the specific LDL receptors, the LDE emulsion was injected into rats treated with 17α-ethinylestradiol, which is known to increase the activity of these receptors; as expected, removal of LDE from the bloodstream increased (FCR = 0.90 ± 0.35 h^{-1}). On the other hand, saturation of the receptors that remove remnants by prior infusion of massive amounts of lymph chylomicrons did not change LDE plasma kinetics. These results indicate that LDE is cleared from plasma by B,E receptors and not by the E receptors that remove remnants. Incorporation of free cholesterol into LDE increased LDE plasma clearance. Incubation studies also showed that LDE incorporates a variety of apolipoproteins, including apo E, a ligand for recognition of lipoproteins by specific receptors. Our data suggest that LDE can be a useful tool to test LDL metabolism and B,E receptor function.

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Low-density lipoprotein (LDL), which carries most of the plasma cholesterol in humans, can be considered a biological microemulsion consisting of a core of cholesteryl esters (45–50% of the particle weight) and residual triglycerides (3–4%) surrounded by a monolayer of phospholipids (16–25%) and unesterified cholesterol (5–8%). The protein moiety of LDL is apolipoprotein (apo) B-100.

LDL is the final product in the lipolysis of very low density lipoprotein (VLDL), a triglyceride-rich lipoprotein synthesized by the liver. VLDL triglycerides are progressively lost from the particles through the action of lipoprotein lipase on the endothelium of capillary vessels of peripheral tissues, such as muscle and adipose tissue. The enzyme is stimulated by a co-factor, apolipoprotein CII, present on the surface of VLDL particles. LDL is removed from plasma by the B,E receptors that recognize the receptor-binding domain of apo B-100. The lipoprotein is then internalized and LDL cholesterol is utilized in various cellular processes (1).

It is possible to model the metabolism of chylomicrons or VLDL with protein-free triglyceride-rich emulsions of defined lipid composition (2–5). In the bloodstream the emulsions acquire apolipoproteins from the circulating lipoproteins undergoing lipolysis. Subsequently, apo E serves as ligand for the binding of the triglyceride-depleted particles to hepatic receptors that take up remnants (E receptors).

In the present study, we tested the hypothesis whether a microemulsion resembling the lipid phase of LDL (6,7), without apo B, could mimic the metabolism of native LDL. The microemulsion (LDE) labeled with radioactive lipids was injected into the bloodstream of control rats to determine the plasma kinetics and the percent uptake of the emulsion by different organs. To confirm the role of the specified LDL receptors in removing LDE from plasma, the LDE plasma kinetics and tissue uptake were also followed in a group of rats treated with 17α-ethynilestradiol, which enhances the activity of these receptors. To test whether the remnant receptors could take up the emulsion, LDE was also injected into rats following infusion of lymph chylomicrons resulting in saturation of these receptors. The effects of hypothyroidism and of LDE supplementation with free cholesterol were also evaluated. Our data show that LDE can be useful to test *in vivo* LDL metabolism and B,E receptor function.

MATERIALS AND METHODS

Preparation of LDE. Egg phosphatidylcholine was purchased from Lipid Products (Surrey, United Kingdom), and triolein, cholesteryl oleate and cholesterol were from Nu-Chek-Prep (Elysian, MN). [4-^{14}C]Cholesteryl oleate, [1α,2α(n)-^{3}H]oleate were from Amersham (Amersham, United Kingdom). Lipids were judged >99% pure by thin-layer chromatography (TLC). The microemulsion utilized in this study was prepared from lipid mixtures composed of 40 mg of egg phosphatidylcholine, 20 mg of cholesteryl oleate and 0.6 mg of triolein, with addition of radioactive lipids. In some experiments, 5% (w/w) cholesterol was also added to the mixture.

The lipid mixtures were sonicated and purified to obtain the microemulsion according to the procedures described by Ginsburg *et al.* (5). Lipid mixtures were dried under an N_2 stream followed by overnight vacuum desiccation at 4°C to remove residual solvent. Dried lipids were resuspended in 10 mL of 0.1 M KCl, 0.01 M Tris HCl at pH 8.0. The suspension was sonicated using a Branson Cell Disruptor model B-30 (São Paulo, Brazil), with 125 Watts output in the "continuous" operating mode, for 180 min under an N_2 atmosphere. The temperature was kept between 53–55°C, as monitored by a thermocouple inserted into the vial. The emulsified lipid suspension was then transferred to clean tubes for ultracentrifugation at 195,000 × *g* (30 min) in an SW 40 rotor of a Beckman

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Abbreviations: AML, acute myeloid leukemia; LDE, microemulsion resembling low-density lipoprotein; LDL, low-density lipoprotein; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

L8-M ultracentrifuge (Palo Alto, CA). The top 10% of the solution, containing particles that float at a background density of approximately 1.006 g/mL, was removed by aspiration with a needle. The remaining solution was adjusted to a background density of approximately 1.22 g/mL by adding solid KBr and was then centrifuged at 195,000 × *g* (120 min) at 4°C. The top 20–30% of the sample was collected by aspiration at room temperature, and dialyzed overnight in buffer to remove KBr from the solution. The microemulsion fraction was analyzed for lipid composition (phospholipids, cholesteryl esters, triacylglycerols) by standard laboratory methods (8–10) and utilized in the experiments described below. Before analysis, the microemulsion containing cholesterol was also submitted to preparative TLC in the solvent system described below to allow separate determination of cholesterol and cholesteryl ester.

Animals. Male Wistar rats, weighing 250–300 g and fed a standard commercial chow (Anderson Clayton, São Paulo, Brazil) *ad libitum*, were utilized in this study. One group of rats was treated with 17α-ethinylestradiol for five days. The drug was dissolved in ethanol (100 μg/μL) and propyleneglycol to a final concentration of 1 mg/mL, and injected subcutaneously at a daily dose of 5 mg/kg of body weight (11). Another group of rats was rendered hypothyroid by addition of propylthiouracil to their drinking water (100 mg/dL) for 30 d (12).

LDE plasma kinetics and tissue uptake. Microemulsions labeled with radioactive core lipids were injected into the bloodstream of control rats and of rats treated with 17α-ethinylestradiol or propylthiouracil to measure the rates of lipid disappearance from plasma.

The animals were anesthetized with diethyl ether, and a polyethylene cannula (Intramedic PE 50) was inserted into the left carotid artery. Clotting inside the cannula was prevented by pretreatment with silicon (Clay Adams, Parsippany, NJ). The animals were kept in individual cages for at least 90 min for recovery from anesthesia. The microemulsion was then injected as a bolus of approximately 3 mg of total lipid, in a volume of approximately 0.5 mL. At intervals of 0.5 g, 1, 2, 4, 7, 9 and 12 h, when the animals were sacrificed by air embolization, blood samples of 0.3 mL were collected into tubes containing 20 μL heparin.

To measure the uptake of LDE by the tissues, the microemulsion was labeled with [1α,2α(n)-^{3}H]cholesteryl oleyl ether. Selected organs were excised 24 h after the injection of the emulsion into three control rats. Plasma samples for determination of emulsion clearance were not taken from these rats.

Determination of radioactivity in plasma and tissues. Lipids were extracted (13) with chloroform/methanol (2:1, vol/vol) from 100 μL aliquots of the separated blood plasma and from approximately 1 g samples of the excised organs. The extracted lipids were concentrated and resolved into classes by TLC using hexane/diethyl ether/acetic acid (70:30:1, by vol) as developing solvent. The cholesteryl ester and triacylglycerol bands were then placed separately into vials with 7 mL scintillation solution [5 g PPO/0.5 g dimethyl POPOP (Sigma, St. Louis, MO)/333 mL Triton X-100/667 mL toluene] (14); and radioactivity was measured by liquid scintillation spectrometry with a LKB model 1211 Spectrometer (Upsala, Sweden).

Competition between LDE and lymph chylomicrons for removal from plasma. Intestinal lymph was collected from male Wistar rats, weighing 300–400 g, over ice with ethylenediaminetetraacetic acid (EDTA) added (final concentration, 1 mM) over 24 h through a cannula implanted into the mesenteric lymph duct. After surgery the rats were maintained in restriction cages, and cottonseed oil was infused (0.035 mL/h) through a gastrostomy tube. Water was accessible *ad libitum.* Saline (5 mL, pH 7.0), d = 1.006 g/mL, containing EDTA (1 mM) was layered on lymph (5 mL) in Beckman SW 40 rotor tubes and centrifuged at 24,500 rpm for 20 min at 20°C (15). Chylomicrons were recovered from 1.5 mL of the creamy top layer aspirated from each tube, and the triglyceride concentration was determined (10). Samples were kept at 4°C and used within 24 h in the experiments.

Lymph chylomicrons were injected into the rats through a carotid cannula, in a bolus corresponding to 7.2 mg of triglyceride every 10 min for 5 h. The labeled microemulsion was injected through the same cannula 5 min after the first injection of lymph chylomicrons. LDE was also injected into three rats in which saline solution (0.8 mg/dL) was substituted for an equivalent volume of lymph chylomicrons (16).

Incubation of LDE with rat plasma high-density lipoprotein (HDL) and with rat plasma HDL apolipoproteins. Microemulsions containing approximately 280 μg of total lipid were incubated with rat HDL (230 μg of HDL protein) obtained by ultracentrifugation of plasma in discontinuous saline gradients (17). Buffer solution (0.01 M Tris-HCl, pH 8.2) was added to attain a 4.0 final volume in each tube. Incubation was for 15 min in a shaking water bath at 37°C. Solid KBr was then added to raise the density to 1.21 g/mL. The mixture was then placed in a Beckman SW41 rotor tube and centrifuged in discontinuous density gradients for 24 h at 20°C (17). The microemulsion particles were recovered in the 1.5 mL top volume of the tube and assayed for protein content (18). Apos associated with the microemulsions were separated by 15% sodium dodecyl sulfate (SDS)-glycerol polyacrylamide gel electrophoresis (19).

Microemulsions (200 μg) were also incubated with rat plasma HDL apo (200 μg), obtained by ultracentrifugation of plasma on discontinuous gradients (17) followed by delipidation of the HDL fraction (20). The incubation, recovery and analysis of the protein associated with LDE were according to the procedures described.

Calculation of LDE removal from plasma. A compartmental model was utilized for the analysis of plasma decaying curves of the emulsion's radioactive lipids (21). The model implies two intravascular (pools 1 and 2) and one extravascular (pool 3) compartment, assuming that the system was in dynamic equilibrium. The emulsion is instantaneously introduced in pool 1. During the elapsed time, a fraction of pool 1 (k_{13}) migrates to pool 3, while another fraction (k_{12}) is transferred to pool 2. A fraction of pool 2 (k_{23}) also migrates to pool 3. Rates of transfer k_{12}, k_{13} and k_{23} were estimated using nonlinear least squares procedures (22,23). Fractional clearance rate (FCR) of the labeled lipids from the intravascular compartment was estimated according to Matthews (24):

$$FCR = \frac{(k_{12} + k_{13}) \cdot k_{23}}{k_{12} + k_{23}} \quad [1]$$

The Student's *t*-test was used to analyze differences between means.

RESULTS

LDE prepared without cholesterol had approximately 63% phospholipids, 36% cholesteryl ester and 1% triacylglycerol, whereas LDE prepared with cholesterol consisted of approximately 61% phospholipids, 35% cholesteryl ester, 2% cholesterol and 3% triacylglycerol. The cholesteryl ester/phospholipid molar ratio was approximately 0.7 in both microemulsions.

When LDE was simultaneously labeled with [4-^{14}C]-cholesteryl oleate and [1α,2α(n)-^{3}H]cholesteryl oleyl ether and injected into four control rats, the removal rate for both labels was nearly identical (FCR = 0.40 ± 0.02 and 0.40 ± 0.04 min^{-1}, respectively). This indicates that practically no recirculation of the LDE cholesteryl ester occurred over 12 h (25). In the rat the cholesteryl ester or ether can indeed be considered the marker of removal of the emulsion from plasma because it is not selectively removed from the emulsion particles (26).

FCR of the LDE cholesteryl ester and triacylglycerols injected in the bloodstream of control, estrogen-treated and hypothyroid rats is shown in Table 1. In control rats, the emulsion triacylglycerols were removed from plasma three times faster than was cholesteryl ester. In the animals treated with estrogen, cholesteryl ester was removed two times faster than in controls, but the removal of triacylglycerols was not affected (Fig. 1). In contrast, treatment with propylthiouracil did not significantly change the removal of cholesteryl ester, but the FCR of triacylglycerols was twice as rapid. The uptake by organs of [^{3}H]cholesteryl ether of emulsions injected in three control rats is shown in Table 2. Most of the emulsion radioactivity was taken up by the liver.

In the rats that were infused lymph chylomicrons, the plasma triacylglycerols raised from 12 to 27 mg/dL 1 h after the beginning of the infusion to 106 mg/dL after 3 h, and 72 mg/dL after 5 h. Figure 2 shows that the clearance of the emulsion cholesteryl ester in these rats (n = 6) was not different from that measured in rats (n = 3) in which the chylomicron infusion was replaced by infusion of an equal volume of isotonic saline solution (0.44 ± 0.19 and 0.51 ± 0.17 h^{-1}, respectively). This suggests that LDE was not removed from the plasma by the mechanisms that remove chylomicron remnants.

When free cholesterol was added to LDE (Fig. 3), FCR of either cholesteryl ester (0.54 ± 0.15 h^{-1}) or triacylglycerols (3.23 ± 0.68 h^{-1}) of the emulsion injected in control rats was significantly increased as compared to LDE

TABLE 1

Effects of Estrogen Treatment and Hypothyroidism on the Removal of Microemulsion Lipids from Plasma[a]

Treatment	Removal from plasma (FCR, h^{-1})	
	Cholesteryl esters	Triacylglycerols
Controls (n = 15)	0.42 ± 0.09	1.40 ± 0.63
Estrogen-treated (n = 10)	0.90 ± 0.35[b]	1.62 ± 0.59
Hypothyroid (n = 7)	0.39 ± 0.09	2.76 ± 0.94[b]

[a]Micromulsions without free cholesterol, and labeled with [4-^{14}C]cholesteryl oleate and glycerol tri[10(n)-^{3}H]oleate were injected into the carotid artery of control rats and rats treated with 17α-ethinylestradiol (5 mg/kg body wt/d during 5 d) or propylthiouracyl (100 mg/dL in drinking water for 30 d). Blood was sampled at regular intervals during 12 h, and the fractional clearance rate (FCR) was calculated from the curves of radioactivity remaining in plasma according to a three-compartmental model. Results are means ± SD.

[b]$P < 0.05$ compared to control values.

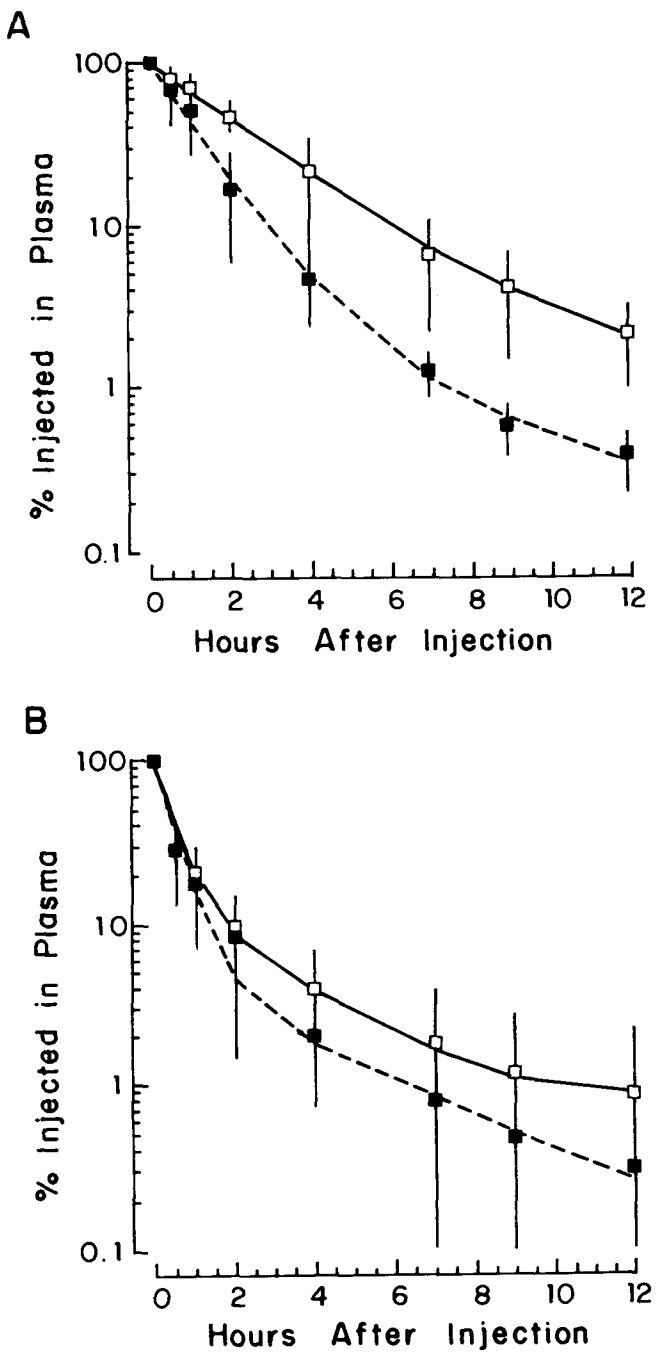

FIG. 1. Removal from plasma of [4-^{14}C]cholesteryl oleate (A) and glycerol tri[10(n)-^{3}H]oleate (B) of the microemulsion in control rats (□) and rats treated with 17α-ethinylestradiol (■).

TABLE 2

Tissue Uptake of the Microemulsion Labeled with [1,2(n)-^{3}H] Cholesteryl Oleyl Ether[a]

Tissue	% of Injected dose	% by Gram of tissue
Liver	83.79 ± 3.98	9.70 ± 1.44
Spleen	1.17 ± 0.25	1.65 ± 0.28
Lung	0.39 ± 0.09	1.73 ± 0.19
Muscle	3.39 ± 1.45	0.03 ± 0.02
Heart	0.11 ± 0.02	0.14 ± 0.03
Kidney	0.13 ± 0.00	0.07 ± 0.01
Adipose	0.74 ± 0.25	0.00 ± 0.00
Adrenal	0.62 ± 0.09	15.52 ± 3.81

[a]The microemulsion did not contain free cholesterol. Tissues were excised for lipid extraction and radioactivity determination 24 h after injection of the emulsion. The results are means ± SD of three experiments.

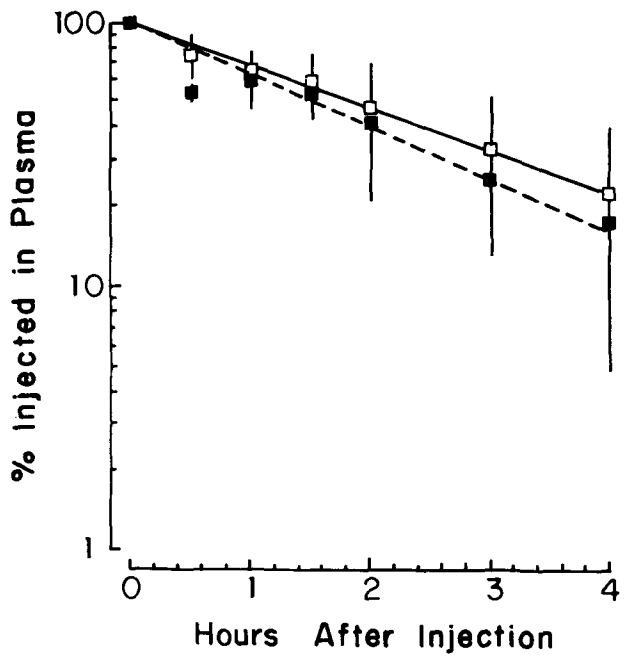

FIG. 2. Removal from plasma of [4-^{14}C]cholesteryl oleate of the microemulsion in rats infused with isotonic saline solution (□) or with lymph chylomicrons (■).

without free cholesterol ($P < 0.05$). FCR of the labeled free cholesterol of the emulsion was 0.92 ± 0.41 h^{-1}.

After incubation with HDL or HDL apo, LDE incorporated apos AI, AIV, E, CII and CIII. As observed in the SDS polyacrylamide gels, no difference was seen in the proportion of incorporated apos in LDE with and without cholesterol.

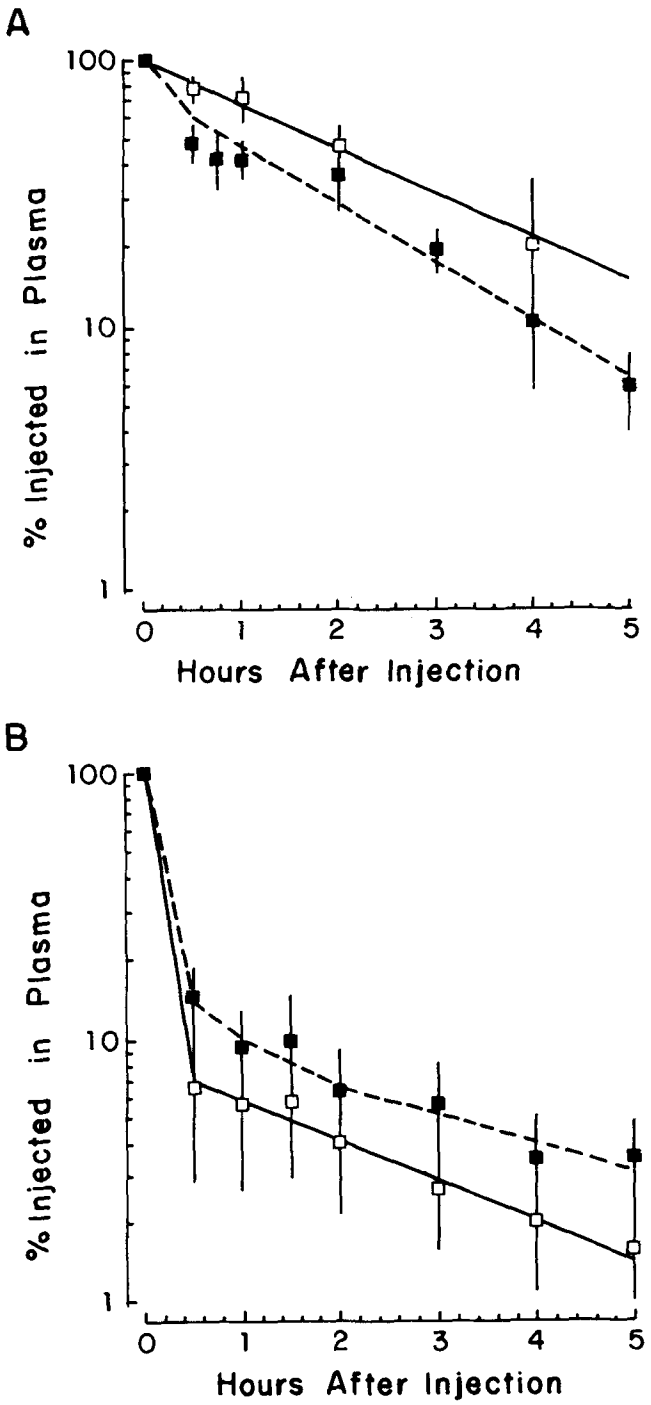

FIG. 3. Removal from plasma of (A) [4-^{14}C]cholesteryl oleate of the microemulsion with (■) or without cholesterol (□) and of (B) [^{14}C]cholesterol (□) and glycerol tri[10(n)-^{3}H]oleate (■) of the microemulsion with cholesterol.

DISCUSSION

The structure of protein-free microemulsions containing egg phosphatidylcholine and cholesteryl oleate, similar to those utilized in the current investigation, has been well-defined previously (5–7). These systems were shown to mimic the lipid portion of native LDL, and incorporation of unesterified cholesterol up to 15% molar did not affect their LDL-like size characteristics (7).

LDL interacts with B,E receptors through apo B-100, but the receptors can also recognize apo E, which is not usually present in the LDL particles. We hypothesized whether LDE, when injected into the bloodstream, could pick up apo E molecules from native lipoproteins, enabling recognition and uptake of the microemulsion by the B,E receptors. In fact, the incubation of LDE with HDL

or HDL apos confirmed that apo E binds the emulsion, together with other exchangeable apos like apos AI and CII and CIII.

The present results show that LDE has a markedly different metabolic behavior in rats as compared to the triglyceride-rich emulsions resembling chylomicrons described in our previous studies (2-4). Whereas the chylomicron-like emulsion was rapidly removed from plasma, LDE was retained for several hours, as native LDL would be. This emphasizes the importance of the lipid portion of these particles for their metabolic behavior, as both emulsions were capable of incorporating the various exchangeable apos present in the plasma. As occurs with native LDL, the liver was the main site of removal of LDE from the circulation. Evidence was gathered here that LDE was taken up by B,E receptors that remove native LDL, instead of by the E receptors that bind chylomicron remnants. Firstly, treatment of the rats with high doses of 17α-ethinylestradiol, which enhances the activity of the B,E receptors severalfold (27,28), resulted in nearly twofold increase in the rate of LDE removal from the plasma. This behavior is identical to that of native LDL injected in estrogen-treated rats (29-31), and different from chylomicron-like emulsions, which were slowly cleared in rats under estrogen treatment (2). Secondly, when LDE was injected in rats infused with amounts of lymph chylomicrons capable of saturating the removal sites of remnants (E receptors) (16), the plasma kinetics of the radioactive cholesteryl ester remained unchanged, suggesting that LDE is not removed by this mechanism.

LDE was also tested in hypothyroidism, a metabolic disorder in which lipoprotein plasma kinetics are well-documented in rats. The results again resembled those of native LDL and were markedly different from the kinetics of chylomicrons and triglyceride-rich emulsions. The plasma clearance of native LDL in hypothyroid rats has been found to be only slightly decreased (32), whereas in our experiments the LDE clearance was unchanged. In contrast, the removal of remnants of chylomicrons and triglyceride-rich emulsions was pronouncedly slowed down (33).

Despite the minimum content of triacylglycerols in LDE, the emulsion underwent lipolysis, as shown by a triacylglycerol FCR greater than that of cholesteryl ester (2,3). Lipolysis was greater in the rats made hypothyroid, probably due to increased lipoprotein lipase activity under this condition (12).

In the current study, we found that addition of free cholesterol to LDE led to acceleration of its removal from plasma. In protein-free lipid model systems for LDL, unesterified cholesterol is located primarily at the particle surface (7). This allows the conjecture that the introduction of cholesterol into LDE could favor the incorporation of apo E into the surface of the microemulsion particles, thus facilitating clearance. Moreover, apo C is known to decrease the binding of apos to receptors, and the addition of free cholesterol could also increase the ratio apo E/C, as previously documented with triacylglycerol-rich emulsions (3,34). However, after incubation with HDL or HDL apos, we found no difference between LDE with and without free cholesterol in regard to the proportion of associated apos.

Plasma kinetics of LDE radioactive, free cholesterol (Fig. 3) were similar to native LDL, as described by Eisenberg *et al.* (35). It is also noteworthy that triglyceride clearance was accelerated by addition of free cholesterol to LDE, which is probably due to enhanced removal of emulsion particles rather than to increased lipolysis.

Extensive studies of LDL plasma kinetics in human subjects could be very useful to understand the pathophysiology of lipid metabolism and atherosclerosis. However, these studies have been restricted by the laborious procedures of isolation and labeling of native LDL and the obligatory use of autologous lipoprotein due to the risk of transmission of HIV or hepatitis virus into recipient subjects. Need of a standard LDL preparation for injection into multiple individuals was already emphasized by Goldstein and Brown in a 1984 review (36) as a requirement for uniform kinetic studies. Our study raises the possibility for the use of LDE as a tool to evaluate LDL metabolism and B,E receptor function. Consistent with this assumption, the rate of removal of LDE from plasma has been shown to be slower in patients with familial hypercholesterolemia, as expected for native LDL (37). In contrast, LDE was rapidly removed from the plasma in patients with acute myeloid leukemia (AML) (38), which is expected because AML cells overexpress B,E receptors and avidly take up the native LDL (39,40). Finally, we would like to suggest that our results provide a rationale for the utilization of LDE to deliver antitumor drugs to neoplastic cells with enhanced expression of LDL receptors.

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Plasma Clearance and Hepatic Utilization of Stearic, Myristic and Linoleic Acids Introduced *via* Chylomicrons in Rats

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The primary objective of the present study was to compare the rates of plasma clearance and hepatic utilization of stearic (18:0), myristic (14:0) and linoleic (18:2) acids, as introduced *via* chylomicrons. Lymph chylomicrons were specifically labeled *in vivo* with [^{14}C]stearic and (SA), [^{14}C]myristic acid (MA), or [^{14}C]linoleic acid (LA) by infusing donor rats intraduodenally with the labeled fatty acids in a lipid emulsion. Following intravenous injection of recipient rats with the labeled chylomicrons, the rates of plasma clearance and incorporation of the label in triglycerides (TG), phospholipids (PL) and other lipids in the liver were compared at 5, 15 and 30 min. [^{14}C]SA was cleared at a slightly faster rate ($t_{1/2}$ = 7.0 min) than [^{14}C]MA ($t_{1/2}$ = 8.1 min) and [^{14}C]LA ($t_{1/2}$ = 8.0 min) ($P < 0.05$). [^{14}C]SA was accumulated in the liver at a significantly faster rate than [^{14}C]MA and [^{14}C]LA. At the peak (15 min) of hepatic uptake, 30.3% of [^{14}C]SA, 26.2% of [^{14}C]LA and 21.9% of [^{14}C]MA were recovered in the liver. At 30 min, 33.5% of [^{14}C]SA was taken up by the liver, whereas 27.8% of [^{14}C]LA and only 15.2% of [^{14}C]MA were removed. In the liver, the percentage of [^{14}C]SA incorporated into PL steadily increased with time, whereas the percent-age incorporated into TG decreased. [^{14}C]SA was preferentially incorporated into PL at all time intervals, as compared with [^{14}C]MA and [^{14}C]LA. At 30 min, 38.6% of [^{14}C]SA was found in PL, and only 5.2% of [^{14}C]MA and 12.0% of [^{14}C]LA were present in PL. A large proportion of hepatic [^{14}C]MA remained unesterified (free fatty acid) throughout the 30-min period, with a small proportion incorporated into PL and TG. Of the total liver ^{14}C radioactivity recovered at 30 min, 63.8% of [^{14}C]MA, 48.8% of [^{14}C]LA and 25.5% of [^{14}C]SA were found unesterified. During 30 min, a significantly greater amount of [^{14}C]MA (76.9%) was oxidized in both the liver and the peripheral tissue combined, compared with [^{14}C]LA (64.7%) and [^{14}C]SA (61.2%). A higher proportion of [^{14}C]LA was incorporated into TG than into PL at all time intervals. No differences were noted in the relative distribution of ^{14}C in cholesterol and other lipids among the three fatty acids. Using labeled fatty acids incorporated *in vivo* into chylomicrons, the present study demonstrated that SA, MA and LA are distinctly different in their metabolic behavior. During the initial 30 min after their entry into the blood, 92–95% of the fatty acids were cleared. During this early phase of metabolism, [^{14}C]SA was preferentially utilized for liver PL synthesis, whereas [^{14}C]LA was better incorporated into TG. [^{14}C]MA was poorly incorporated into hepatic lipids, but was preferentially oxidized in the liver or utilized by the peripheral tissue.
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Compared with polyunsaturated fatty acids, such as linoleic acid (LA), dietary long-chain saturated fatty acids generally have been regarded as hypercholesterolemic. However, unlike other saturated fatty acids, such as myristic acid (MA) and palmitic acid (PA), stearic acid (SA) lowers plasma cholesterol and is less atherogenic, as has been demonstrated in various animal models (1). The proposed mechanisms for its hypocholeterolemic effect include (i) incomplete digestion of fats high in SA and its inefficient intestinal absorption (2,3); (ii) reduced intestinal absorption of cholesterol with diets high in SA (2); and (iii) its metabolic conversion to oleic acid (OA) by desaturation (4–6).

At present, little information exists concerning how SA may differ from other fatty acids, especially when introduced into the circulation *via* chylomicrons, which are the principal carriers of dietary fats. In a recent study (7), we compared the rates of plasma clearance and hepatic metabolism of SA, PA and OA in rats after intravenous injection of chylomicrons labeled *in vivo* with the fatty acids. The findings from this study (7) suggest that the hypocholesterolemic effect of SA is not associated with its conversion to oleic acid, but with its preferential utilization for phospholipid (PL) synthesis. PA, on the other hand, is most rapidly removed from the plasma and incorporated into triglyceride (TG) in the liver. This observation suggests that PA is more stimulatory to hepatic lipogenesis and, hence, the synthesis of very low density lipoprotein (VLDL) contributing to hyperlipemia. The results also indicate that OA is more readily taken up by the peripheral tissue and/or oxidized in the liver.

The present study was undertaken to compare the metabolic fates of SA, MA and LA immediately after their transport into the circulation *via* chylomicrons. We prepared chylomicrons enriched and radiolabeled *in vivo* with these fatty acids, instead of using free fatty acids (FFA) (8). The rates of plasma clearance and hepatic utilization of these fatty acids were compared after intravenous injection of the labeled chylomicrons.

MATERIALS AND METHODS

Animals and diets. Fifty-one male albino rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), weighing 266 ± 16 g, were housed individually in stainless-steel cages and subjected to a cycle with a 1500–0300 light period and a 0300–1500 dark period. Temperature and humidity were controlled at 23–25°C and 55–75%, respectively. The animals were cared for in an animal care facility accredited by the American Association for the Accreditation of Laboratory Animal Care. The rats were fed a nutritionally adequate diet, formulated according to the recommendations (9,10) of the American Institute of Nutrition (AIN), except for its fat content. The diet, as shown in Table 1, contained 5% beef tallow in addition to the 5% corn oil recommended by the AIN. This modification was made to better simulate the amount and type of dietary fat present in a typical American diet. All animals were fed the diet *ad libitum* and allowed free access to deionized water throughout the experiment.

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Abbreviations: FFA, free fatty acid; LA, linoleic acid; LDL, low-density lipoprotein(s); LPL, lipoprotein lipase; MA, myristic acid; OA, oleic acid; PA, palmitic acid; PL, phospholipid; SA, stearic acid; TLC, thin-layer chromatography; TG, triglyceride; VLDL, very low density lipoproteins.

TABLE 1

Composition of Experimental Diet

Ingredient	g/kg
Egg whites, spray dried	200
Corn starch	100
Dextrose	502.996
Cellulose	50
Corn oil	50
Beef tallow	50
Salt mix[a]	35
Vitamin mix[a]	10
Biotin	0.004
Choline bitartrate	2

[a]According to the recommendations of the American Institute of Nutrition (Refs. 9 and 10).

Cannulation of mesenteric lymph duct. In order to obtain chylomicrons labeled with specific fatty acids, the mesenteric lymph duct was cannulated by a modification of the procedure described in our previous studies (11,12). At the end of 8 wk, the rats were fasted for 24 h before surgery. Under halothane anesthesia, an abdominal incision was made along the midline with a cauterizer. The major intestinal lymph duct was cannulated with vinyl tubing (SV. 31 tubing; 0.50 mm i.d., 0.80 mm o.d. Dural Plastics, Auburn, Australia). Following the cannulation, an indwelling infusion catheter (Silastic medical grade tubing; 1.0 mm i.d., 2.1 mm o.d., Dow Corning, Midland, MI) was inserted *via* the gastric fundus into the upper duodenum and secured by a purse-string suture (4-0 Silk; Ethicon Inc., Somerville, NJ). After the abdominal incision was closed, the rats were placed in restraining cages (13) in a heated chamber (30°C) for 24 h to prevent hypothermia after surgery. During this postoperative recovery period, the rats were infused (Model 5B, Auto-syringe Inc., Londonderry Turnpike, Hookset, NH) *via* the duodenal catheter with a maintenance solution consisting of 5% glucose, 0.87% NaCl and 0.03% KCl at the rate of 2.5 mL/h. The volume of lymph ranged from 1.8 to 2.2 mL/h.

Preparation and labeling of chylomicrons. Chylomicrons were labeled *in vivo* with a [1-^{14}C]fatty acid as follows. Six lymph-fistula rats weighing 432 ± 32 g were divided into three groups. Each of the donor rats was infused *via* the duodenal catheter with a lipid emulsion (infusate) containing SA, MA and LA. The infusate was obtained by mixing 18–20 mL of Intralipid (10% soybean oil, 1.2% egg yolk phospholipid and 2.25% glycerin, USP, KabiVitrum, Alameda, CA) containing 10 mg α-tocopherol and an emulsion of SA, MA and LA prepared by sonication in 0.75 g Na-taurocholate in 40–42 mL phosphate buffered saline (6.75 mM Na_2HPO_4, 16.5 mM NaH_2PO_4, 115 mM NaCl, 5 mM KCl and 2.5 g glucose, pH 6.4). The total concentrations of SA, MA and LA in the mixture were 0.74, 0.58 and 0.58 g per 60 mL, respectively. More SA was added to the infusate because of its relatively inefficient intestinal absorption. After addition of 15–25 μCi of the specific [1-^{14}C]-labeled SA, MA or LA, the infusate was sonicated for 10 min to obtain uniform mixing of the label (W-375; Heat Systems-Ultrasonics, Long Island, NY). The radiochemical purities of all ^{14}C-labeled fatty acids (NEN, Du Pont, Wilmington, DE; specific activities, 56–58 mCi/mmol) were 98.9–99.0% by thin-layer chromatography (TLC).

TABLE 2

Distribution (%) of Radioactivity Among Different Lipid Classes in Chylomicrons Produced by Infusing Lipid Emulsions[a]

	PL	MG	DG + CH	FFA	TG	CE
MA	0.8	0.0	1.5	0.8	96.7	0.3
LA	3.8	0.4	4.8	0.0	92.1	0.8
SA	3.4	0.0	0.5	0.4	92.3	3.4

[a]PL, phospholipid; MG, monoglyceride; DG + CH, diglyceride and cholesterol; FFA, free fatty acid; TG, triglyceride; and CE, cholesteryl ester. Chylomicrons labeled with MA, [^{14}C]myristic acid; LA, [^{14}C]linoleic acid; and SA, [^{14}C]stearic acid.

After a steady flow of lymph was established during the postoperative recovery period, the donor rats were infused *via* the duodenal catheter with the respective lipid emulsion at 2.5 mL/h. Lymph was collected for about 20 h at 30°C in a sterilized 50-mL plastic tube. The lymph was defibrinated by passing through glass wool. The filtrate was overlayered with 150 mM NaCl (pH 7.4) in polyallomer tubes and centrifuged at $1.3 \times 10^6 \times g$/min at 28°C using a Beckman 50.3 Ti rotor in a Beckman L5-75B ultracentrifuge (Spinco Division, Palo Alto, CA). The packed top fraction of chylomicrons was separated and suspended in 150 mM NaCl, pH 7.4, by passage through a 23-gauge hypodermic needle. The dispersed chylomicrons were preheated at 55–60°C for 2 min under N_2 and cooled to 37°C prior to injection. Previously (14), it was found that reheating to 58°C was necessary to restore the spherical shape of the lymph lipoproteins rich in saturated fat exposed to temperatures of 23–26°C. Approximately 92–97% of the ^{14}C radioactivity of the chylomicrons was associated with TG, as determined by TLC (Table 2). The fatty acid composition of the chylomicrons closely reflected that of the duodenal infusates (Table 3), as analyzed by gas chromatography (Hewlett-Packard Model 5880A Gas Chromatograph, Hewlett-Packard, Palo Alto, CA).

Determination of the rate of plasma clearance. Forty-five recipient rats were divided into three groups with 15 rats each. After fasting for 10 h, each of the three groups was injected *via* the jugular vein with a dose of chylomicrons labeled with [^{14}C]SA, [^{14}C]MA or [^{14}C]LA. The

TABLE 3

Fatty Acid Compositions (%) of Lipid Emulsions Enriched with Myristic, Linoleic or Stearic Acid, and Chylomicrons Produced by Infusing the Lipid Emulsions (infusates)[a]

		Fatty acid					
		14:0	16:0	18:0	18:1	18:2	18:3
Infusate							
	MA	23.9	7.2	26.0	12.5	27.1	3.2
	LA	19.8	7.8	32.3	14.6	23.2	2.1
	SA	19.3	6.7	32.8	12.2	26.1	2.9
Chylomicrons							
	MA	25.5	12.7	22.3	12.0	25.1	2.3
	LA	19.1	10.0	23.3	14.4	30.1	3.2
	SA	21.3	11.3	23.1	14.1	27.9	2.4

[a]Infusates labeled with MA, [^{14}C]myristic acid; LA, [^{14}C]linoleic acid; and SA, [^{14}C]stearic acid. Chylomicrons labeled with MA, LA and SA.

average body weights for the SA, MA and LA groups were 403 ± 18, 406 ± 16 and 400 ± 16 g, respectively. A dose of chylomicrons labeled with [^{14}C]SA, [^{14}C]MA or [^{14}C]LA contained 13.5, 10.2 and 10.2 mg of TG, respectively. Five rats from each group were killed under diethyl ether anesthesia at 5, 15 and 30 min after dosing. These specified time intervals (5, 15 and 30 min) were chosen because of the rapid rate of clearance of chylomicron fatty acids from the plasma. Blood samples (2 mL) were collected *via* the orbital sinus (15), and plasma was separated by centrifugation at 1000 × *g* for 60 min. Aliquots (200 μL) of plasma were mixed with scintillation liquid (ScintiVerse, Fisher Scientific, Fairlawn, NJ) and counted to determine the plasma ^{14}C radioactivity (Beckman LS 8000; Beckman Instruments, Fullerton, CA). Total volume of plasma was estimated at 3.35% of the body weight as determined by a radioisotopic dilution method (16). Percent clearance of the injected dose at each interval was computed following the equation (^{14}C dpm at t_i/^{14}C dpm at t_0) × 100; where t_i is a given time interval and t_0 is time of dosing. The rate of clearance for each fatty acid was expressed by half life ($t_{1/2}$), which is the time taken for the ^{14}C radioactivity to decrease to one-half of the total amount injected. The $t_{1/2}$ was determined by the equation for the rate of an exponential change, $t_{1/2} = -0.693/k$; where k is the slope of the time-course clearance curve of ^{14}C radioactivity.

Hepatic uptake and utilization of ^{14}C fatty acids in different lipid classes. After exsanguination of rats under halothane anesthesia, livers were removed, blotted with absorbent paper, weighed and frozen at −70°C until analysis. The whole liver was finely minced with a razor blade. A 2-g sample was taken and used for extraction of lipids by the method of Folch *et al.* (17). The total ^{14}C radioactivity taken up by the liver was determined by counting an aliquot (250 μL) of liver lipid extract and expressed as percent of the dose injected. Distribution of ^{14}C radioactivity in different lipid classes was determined by separating the liver lipids by TLC on silica gel G (20 × 20 cm, 250 μ; Analtech, Newark, DE) with *n*-hexane/diethyl ether/glacial acetic acid (70:30:2, by vol). The solvent system gave an excellent separation of cholesteryl ester, TG, FFA, monoglyceride and PL, except for cholesterol and diglyceride, which comigrated and were collected as one fraction. Separated lipids were visualized with iodine vapor. The lipid spots on TLC plates were scraped into counting vials and eluted with 1.0 mL of 100% ethanol for 10 min prior to mixing with scintillation liquid (ScintiVerse, Fisher Scientific). The distribution (%) of ^{14}C radioactivity among liver lipids was calculated. The fatty acid composition of liver lipids also was determined by gas chromatography.

Distribution of ^{14}C radioactivities of chylomicrons and liver lipid extracts between saturated and unsaturated fatty acids. The chylomicron and liver lipids were further saponified and methylated using methanolic BF_3 as described by Slaver and Lanza (18). Methyl esters of saturated and unsaturated fatty acids were separated by silver-nitrate impregnated TLC (silica gel G impregnated with 10% $AgNO_3$, 20 × 20 cm, 250 μ; Analtech). The plates were developed in 100% chloroform, and lipid fractions were visualized by spraying with water. The separated lipids were scraped into counting vials, eluted with 100% ethanol and counted to determine ^{14}C radioactivity.

Statistics. Analysis of variance with the least significant difference test of the SAS statistical package was used to determine differences ($P < 0.05$) between group means. All data were expressed as mean ± SD.

RESULTS

Comparison of the rates of plasma clearance. Figure 1 shows the time-course ^{14}C-clearance curves for the labeled fatty acids. The rates of ^{14}C-clearance ($t_{1/2}$) during the 30-min period for SA, MA and LA were 7.0 ± 0.3, 8.1 ± 1.2 and 8.0 ± 0.3 min, respectively. [^{14}C]SA was removed at a slightly faster rate ($P < 0.05$) than [^{14}C]MA and LA. Initially (at 5 min), there was no significant difference in clearance rates among the three fatty acids. At 15 min, 75% of [^{14}C]SA, 64% of [^{14}C]LA and 53% of [^{14}C]MA were cleared. At 30 min, 92–95% of the fatty acids was removed from the plasma. The plasma ^{14}C radioactivities of both SA and LA decreased almost linearly with time. The ^{14}C radioactivity of MA, however, was cleared in a curvilinear fashion. The clearance curve for [^{14}C]MA was characterized by an initial slow phase followed by a rapid phase of clearance.

Comparison of hepatic and extrahepatic uptake. Figure 2 compares the time course changes in the ^{14}C radioactivities recovered in the liver. The hepatic ^{14}C radioactivities, as expressed in percent dose, generally reflected the amounts of the ^{14}C cleared from the plasma. The hepatic ^{14}C radioactivities increased rapidly with time up to 15 min and plateaued at 30 min, except for [^{14}C]MA, which decreased significantly from 15 min, despite its rapid removal from the plasma. Between 15 and 30 min, the liver radioactivity of [^{14}C]MA decreased by 6%, suggesting a faster rate of oxidation in the liver. At 15 min,

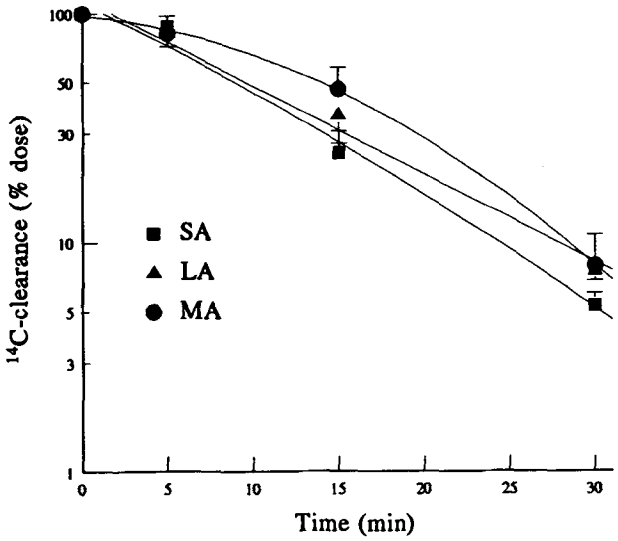

FIG. 1. Clearance of ^{14}C-labeled stearic, myristic and linoleic acids from plasma. Clearance rate (half time, $t_{1/2}$) was determined by the equation $t_{1/2} = -\ln 2/k = -0.692/k$; where k is the slope of the clearance curve. The rates of ^{14}C clearance for stearic, myristic and linoleic acids were 7.0 ± 0.3, 8.1 ± 1.2 and 8.0 ± 0.3 min, respectively. [^{14}C]Stearic acid (SA) was cleared at a slightly faster rate ($P < 0.05$), as compared with [^{14}C]linoleic acid (LA) and [^{14}C]myristic acid (MA) (n = 5 each data point).

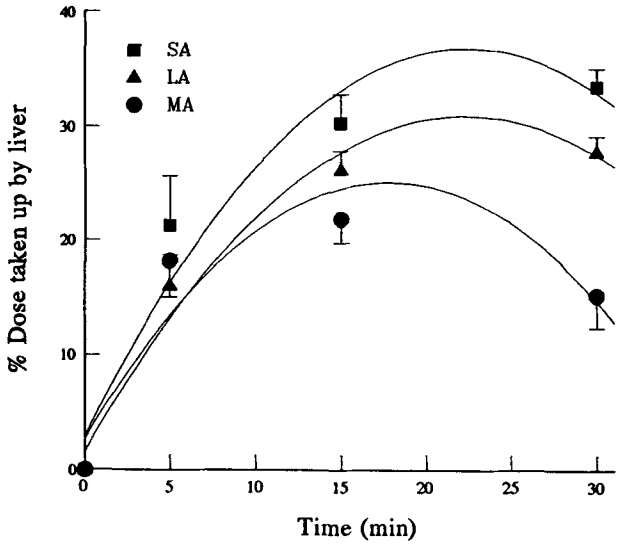

FIG. 2. Time-dependent changes (% dose) in ^{14}C radioactivity in the liver after intravenous injection of chylomicrons labeled with ^{14}C-labeled stearic, linoleic and myristic acids (n = 5 each data point). At 15 and 30 min, significantly greater percentages of the [^{14}C]SA dose were taken up by the liver, compared with [^{14}C]LA and [^{14}C]MA. At the same intervals, a greater percentage of the [^{14}C]LA dose appeared in the liver, as compared with [^{14}C]MA. The radioactivities of [^{14}C]LA and [^{14}C]SA increased linearly with time and plateaued at 30 min, whereas the radioactivity of [^{14}C]MA increased up to 15 min, but precipitously declined at 30 min. Abbreviations as in Figure 1.

30.3% of [^{14}C]SA, 26.2% of [^{14}C]LA and 21.9% of [^{14}C]MA were removed by the liver. At 30 min, 33.5% of [^{14}C]SA, 27.8% of [^{14}C]LA and 15.2% of [^{14}C]MA were recovered.

The loss of ^{14}C radioactivity was calculated as the initial ^{14}C dose minus the ^{14}C radioactivities recovered from the liver and remaining in the plasma at each time interval (Table 4). Thus, the difference was attributable to the ^{14}C taken up by the peripheral tissue and the fraction of the liver ^{14}C radioactivity lost through oxidation. As determined at 30 min, a significantly higher amount of [^{14}C]MA (76.9%) than [^{14}C]LA (64.7%) and [^{14}C]SA (61.2%) was attributable to peripheral uptake and oxidation.

The percentages of the ^{14}C dose appearing in different liver lipids are shown in Table 5. For all fatty acids, less than 3.7% of the ^{14}C dose was present in the combined fractions of monoglyceride, diglyceride and free and esterified cholesterol. Much of each injected ^{14}C dose was associated with FFA (unesterified), TG and PL. The time course appearance of ^{14}C-fatty acids in these lipids in the liver, as expressed in percent dose, is presented graphically in Figure 3. The upper panel compares the [^{14}C]SA, [^{14}C]MA and [^{14}C]LA appearing as FFA. Among the three fatty acids, [^{14}C]MA accumulated most rapidly as FFA in the liver. At 5 min, almost 15% of the dose of [^{14}C]MA was recovered in the FFA fraction. The unesterified [^{14}C]MA increased to 17% at 15 min and decreased to 10% of the dose at 30 min. Between 5 and 15 min, the free [^{14}C]MA represented 78-82% of the total hepatic [^{14}C]MA radioactivity. In contrast, the radioactivities of

TABLE 4

Percentage of the ^{14}C Dose Attributable to Peripheral Uptake and Hepatic Oxidation at Different Time Intervals After Injection of the Chylomicrons Labeled with [^{14}C]Myristic, [^{14}C]Linoleic or [^{14}C]Stearic Acids[a]

	Time after injection of ^{14}C fatty acid		
	5 min	15 min	30 min
MA	0[b]	31.5 ± 13.1[b]	76.9 ± 5.1[b]
LA	4.8 ± 5.8[b]	37.8 ± 8.6[b,c]	64.7 ± 2.0[c]
SA	0[b]	45.2 ± 4.9[c]	61.2 ± 1.3[c]

[a]Calculated by 100% − (% dose in liver + % dose remaining in plasma). This difference was attributed to ^{14}C taken up by the peripheral tissue and the fraction of the liver ^{14}C radioactivity lost by oxidation. Chylomicrons labeled with MA, [^{14}C]myristic acid; LA, [^{14}C]linoleic acid; and SA, [^{14}C]stearic acid.
[b,c]Mean ± SD, n = 5. Values not sharing common superscripts within the same column are significantly different ($P < 0.05$).

TABLE 5

Percentage of ^{14}C Dose Radioactivity Appearing in Monoglyceride, Diglyceride plus Cholesterol, and Cholesteryl Ester Fractions in the Liver at Different Time Intervals After Injection of Chylomicrons Labeled with [^{14}C]Myristic, [^{14}C]Linoleic or [^{14}C]Stearic Acid[a]

	MG	DG + CH	CE
5 min			
MA	0.1 ± 0.1[b]	0.9 ± 0.5[b]	0.0 ± 0.0[b]
LA	0.2 ± 0.1[b]	1.1 ± 0.3[b]	0.1 ± 0.1[b]
SA	0.5 ± 0.4[b]	1.8 ± 0.8[b]	0.5 ± 0.4[b]
15 min			
MA	0.1 ± 0.1[b]	1.1 ± 0.5[b]	0.1 ± 0.1[b]
LA	0.4 ± 0.1[c]	1.9 ± 0.5[c]	0.2 ± 0.1[b]
SA	1.0 ± 0.5[c]	2.3 ± 0.4[c]	0.4 ± 0.2[b]
30 min			
MA	0.1 ± 0.1[b]	0.8 ± 1.0[b]	0.1 ± 0.1[b]
LA	0.8 ± 0.3[c]	1.4 ± 0.3[b]	0.4 ± 0.2[b]
SA	1.2 ± 0.8[c]	1.8 ± 0.5[b]	0.7 ± 0.6[b]

[a]Mean ± SD, n = 5. Values not sharing common superscripts within the same lipid class are significantly different ($P < 0.05$). MG, monoglyceride; DG + CH, diglyceride plus cholesterol; and CE, cholesteryl ester. Chylomicrons labeled with MA, [^{14}C]myristic acid; LA, [^{14}C]linoleic acid; and SA, [^{14}C]stearic acid.

free [^{14}C]SA and [^{14}C]LA remained significantly lower at all time intervals. The radioactivity of free [^{14}C]SA increased slowly with time from 6.1 to 8.6% of the dose, despite its faster clearance from the plasma and uptake by the liver. The radioactivity of free [^{14}C]LA tended to rise more rapidly and plateaued at 15 min, but decreased to 7.3% at 30 min.

At all time points, [^{14}C]MA was minimally utilized for the synthesis of TG, whereas [^{14}C]SA and [^{14}C]LA were incorporated into TG in significantly greater proportions (middle panel, Fig. 3). Initially, both [^{14}C]SA and [^{14}C]LA were rapidly utilized for TG synthesis at a similar rate. The incorporation of the fatty acids into TG reached a maximum at 15 min and tended to plateau thereafter. At 30 min, 13.6% of [^{14}C]LA and 10.5% of [^{14}C]SA were incorporated in TG, whereas only 3.8% of [^{14}C]MA was found in this lipid. Among the three fatty acids, the incorporation (percent dose) of [^{14}C]SA into PL was most

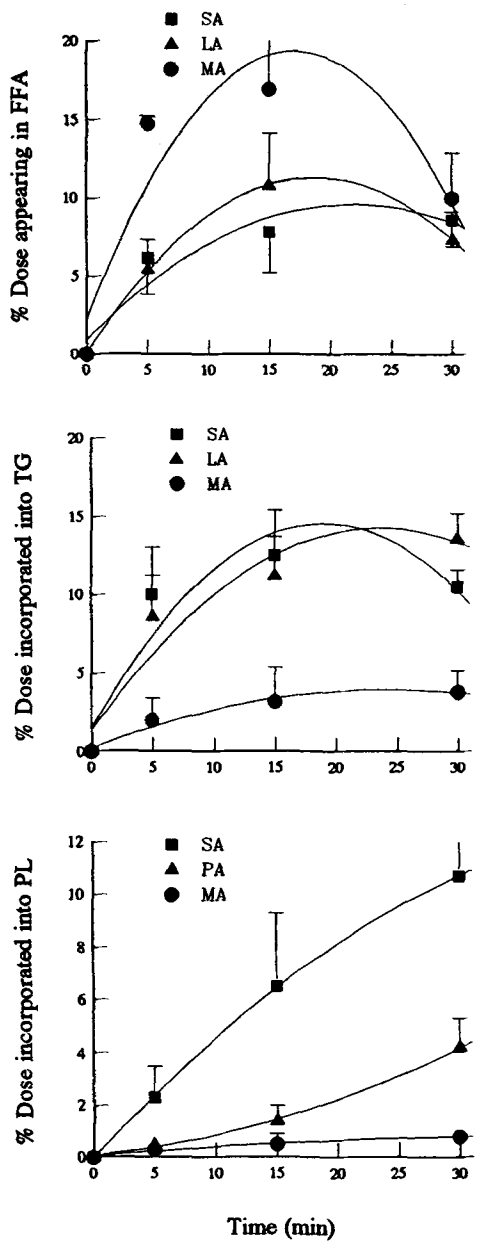

FIG. 3. Time course changes (% dose) in the ^{14}C radioactivity appearing in different lipid classes of the liver after intravenous injection of chylomicrons labeled with ^{14}C-labeled stearic, myristic and linoleic acids (n = 5 each data point). Upper panel: ^{14}C radioactivity (% dose) appearing in free (unesterified) fatty acid (FFA). At 5 min, a significantly greater percentage of [^{14}C]MA appeared as FFA, compared with [^{14}C]LA and [^{14}C]SA. The unesterified [^{14}C]MA remained elevated up to 15 min, but declined precipitously to the levels of [^{14}C]LA and [^{14}C]SA at 30 min. Middle panel: ^{14}C radioactivity (% dose) appearing in triglyceride (TG). No difference was observed between [^{14}C]LA and [^{14}C]SA incorporated into TG up to 15 min, with a slight increase in [^{14}C]LA in TG at 30 min. [^{14}C]MA was most slowly incorporated into TG. At any given interval, less than 4% of [^{14}C]MA dose appeared in TG. Lower panel: ^{14}C radioactivity (% dose) appearing in phospholipid (PL). At all time points, a significantly greater incorporation of [^{14}C]SA into PL was noted, compared with [^{14}C]LA and [^{14}C]MA. No difference between [^{14}C]LA and [^{14}C]MA was observed up to 15 min. At 30 min, a significantly greater percentage of [^{14}C]LA was found in PL compared with [^{14}C]MA. The incorporation of [^{14}C]MA did not change appreciably with time. Less than 1.0% of [^{14}C]MA dose appeared in PL at any time interval. Abbreviations as in Figure 1.

rapid and increased sharply with time (lower panel, Fig. 3). The incorporation of [^{14}C]LA into PL increased linearly with time, but at a much slower rate than that of [^{14}C]SA. [^{14}C]MA was poorly utilized for PL synthesis in the liver. At 30 min, 10.7% of [^{14}C]SA and 4.2% of [^{14}C]LA were found in PL, whereas only 0.8% of [^{14}C]MA was incorporated into this lipid.

Interconversion between ^{14}C fatty acids. The distribution of ^{14}C radioactivity of chylomicrons and hepatic lipids between saturated and unsaturated fatty acids is shown in Table 6. More than 93% of [^{14}C]SA and 95% of [^{14}C]MA were distributed in the saturated fatty acid fraction in both chylomicrons and liver lipids, indicating that 5-7% of these fatty acids were desaturated in the liver. All [^{14}C]LA was found in the unsaturated fatty acid fraction.

DISCUSSION

Several previous studies have compared the metabolic fates and utilization of various fatty acids in intact animals (8), as well as in isolated livers (19,20) and hepatocytes *in vitro* (21). Using chylomicrons labeled *in vivo* with [^{14}C]MA, [^{14}C]SA and [^{14}C]LA, we compared the rates of their plasma clearance and hepatic utilization. The present study was designed specifically to examine the metabolic patterns of these fatty acids immediately after their entry into the circulation *via* chylomicrons, the principal carriers of dietary fatty acids.

The results of this study showed that: (i) SA is removed from the plasma at a slightly but significantly faster rate than MA and LA; (ii) once taken up by the liver, SA is preferentially incorporated into PL; (iii) LA is better utilized for the synthesis of TG than PL; (iv) MA remains largely unesterified in the liver during the early stage of its metabolism and is poorly utilized for lipid synthesis; and (v) with time, MA is more readily oxidized by the peripheral tissue and the liver.

Much of the fatty acids incorporated into chylomicron TG are released during lipolysis by lipoprotein lipase (LPL) on the endothelial surface of the peripheral tissue (22). Chylomicron remnants with residual TG are enriched in apo-E and rapidly removed by the liver *via* the apo-E (chylomicron remnant) receptor (23). Because the bulk of the total fatty acids is present in TG, the rate of plasma clearance of a chylomicron fatty acid is largely determined by the rates of both peripheral lipolysis and hepatic removal. The present data showed that, during the early stage of chylomicron metabolism (up to 15 min), [^{14}C]SA and [^{14}C]LA were removed at a similar rate, but more rapidly than [^{14}C]MA, suggesting a slower rate of peripheral release of [^{14}C]MA from chylomicron TG. With time, however, the clearance of [^{14}C]MA was accelerated, and its plasma radioactivity declined to the level of [^{14}C]LA. These observations suggest that SA and LA are first liberated by LPL from chylomicron TG. As these fatty acids are removed, glycerides containing MA are cleared at progressively accelerated rates.

The hepatic uptake of the fatty acids increased sharply immediately after the labeled chylomicrons were injected and plateaued at 15 min, reflecting their initial rates of plasma clearance. After being taken up by the liver, the fatty acids were distinctly different in their metabolic patterns. [^{14}C]SA was most preferentially incorporated into

TABLE 6

Distribution (%) of ^{14}C Radioactivities of Injected Chylomicron and Liver Lipids Between Saturated and Unsaturated Fatty Acids[a]

	Chylomicrons		Liver removed at 5 min		Liver removed at 15 min		Liver removed at 30 min	
	SAT	UNSAT	SAT	UNSAT	SAT	UNSAT	SAT	UNSAT
MA	99.4	0.6	95.9 ± 2.1	4.1 ± 2.1	98.8 ± 0.8	1.2 ± 0.8	95.4 ± 4.9	4.6 ± 4.9
LA	0	100	0	100	0	100	0	100
SA	99.0	1.0	97.8 ± 2.1	2.2 ± 2.1	98.3 ± 0.8	1.7 ± 0.8	93.8 ± 3.8	6.2 ± 3.8

[a]SAT, saturated; UNSAT, unsaturated. Chylomicrons labeled with MA, [^{14}C]myristic acid; LA, [^{14}C]linoleic acid; and SA, [^{14}C]stearic acid.

PL (Fig. 3), as was also observed in our previous study (7). The present data showed that the [^{14}C]SA radioactivity in PL steadily increased, with a reciprocal linear decline in [^{14}C]SA present in TG. This indicates that SA released from chylomicron remnant TG is continually channeled into PL synthesis. Previously, Leyton *et al.* (8) compared the metabolic utilization of various fatty acids after oral administration of ^{14}C-labeled fatty acids to fed weanling rats. At 24 h after dosing, a significantly greater percentage of [^{14}C]SA was found in liver PL, as compared with [^{14}C]MA and [^{14}C]LA. Elovson (24) also observed a rapid incorporation of [^{14}C]SA into PL in the rat liver. In this study (24), nearly 15% of the fatty acid was recovered in PL at 30 s after intraportal injection of the labeled fatty acid bound to albumin. In addition, SA was found to be oxidized most slowly among the saturated fatty acids with chain lengths ranging from C_{12} through C_{18} (8). The rates of oxidation decreased with increasing chain length. At 1 h, 0.3% of the oral dose of [^{14}C]SA was expired as $^{14}CO_2$, compared with 3.1% from [^{14}C]MA (8). In the present study using fasted rats, we also found that a significantly lower percentage of SA (61%) than MA (77%) was oxidized during a 30-min period.

The above observations, taken together, indicate that SA is more slowly oxidized, but more efficiently utilized for PL synthesis than is MA. Much (63–80%) of the liver [^{14}C]MA remained unesterified. Among the three fatty acids, unesterified [^{14}C]MA accumulated most rapidly in the liver, even during the first 15 min. This initial rapid accumulation occurred despite the significantly lower hepatic uptake of [^{14}C]MA from the plasma, suggesting that the MA present in chylomicron remnant TG is more rapidly liberated in the liver. Compared with [^{14}C]SA and [^{14}C]LA, a significantly greater percentage of the unesterified [^{14}C]MA was lost by oxidation in the liver. This was evidenced by a rapid rise followed by a precipitous decline in free [^{14}C]MA at 30 min, with minimal incorporation into TG or PL (Fig. 3). Thus, free MA is largely oxidized and minimally utilized for lipid synthesis. The preferential incorporation of SA into PL strongly suggests that much of the hepatic SA is destined for incorporation into cellular membranes and lipoprotein surface coats. Such metabolic preference may partly explain the hypolipidemic effect of SA compared with that of other saturated fatty acids (1). However, the rapid oxidation and minimal incorporation of MA into hepatic lipids is not consistent with its well-known hypercholesterolemic effect (1,25,26). Previous research has suggested that MA lowers the turnover of sterols and stimulates the intestinal absorption of cholesterol (27). Also, it has been suggested that MA may suppress expression of the low-density lipoprotein (LDL) receptor (21).

Compared with SA, LA appeared more slowly into the liver. This was partly due to its slower uptake and perhaps to a faster rate of oxidation within the liver after its uptake. Proportionally, more of the hepatic LA was incorporated into TG (31.6–33.0%) than into PL (7.0–12.0%). Compared with [^{14}C]SA, the [^{14}C]LA radioactivity in TG continued to increase with time and significantly exceeded the amount of [^{14}C]SA incorporated into TG at 30 min. On the other hand, the radioactivity of [^{14}C]LA appearing in PL reached only 39% of that of [^{14}C]SA in PL. The difference in the relative incorporation of the label in TG or PL was not related to a difference in intrahepatic dilution of the labeled fatty acids. The percent distributions of total SA and LA, as determined at 30 min, were 20.0 ± 1.9 and 17.4 ± 1.1%, respectively. Our observation is in agreement with the previous finding of an increased appearance of orally-fed [^{14}C]LA in hepatic TG (8). This suggests that dietary LA, as transported *via* chylomicrons, is largely repackaged into TG in the liver, and may accelerate VLDL synthesis. Consistent with these findings, Ohtani *et al.* (28) showed that the secretion of VLDL was markedly increased in cultured hepatocytes from rats fed 5% dietary LA. In addition, using perfused rat livers, Wilcox *et al.* (29) had found that the total output of TG was significantly greater when the livers were perfused with LA, as compared with saturated fatty acids such as SA and MA. The VLDL particles secreted in response to LA or other unsaturated fatty acids contained relatively more TG and less cholesterol (29). Therefore, the observations suggest that the hypocholesterolemic effect of LA may not be associated with a suppression of VLDL synthesis (30), but primarily with a faster catabolism of VLDL enriched with LA. Available evidence, indeed, indicates that VLDL produced by feeding LA-rich fats are more efficiently removed (19,31), and LDL is more rapidly taken up from the plasma, perhaps because of its increased fluidity (20,32). The increased catabolic rate of plasma LDL also may be associated with an increase in LDL-receptor activity by high intake of dietary LA (33–35).

In summary, the present findings clearly illustrate the existence of distinct metabolic patterns for chylomicron fatty acids with different chain lengths and degrees of saturation. SA, MA and LA differ metabolically with respect to their rates of plasma clearance and the extents of their utilization as the substrates for specific lipid

synthesis. The present results confirm our previous finding (7) that SA, as transported in the form of chylomicron lipids, is more rapidly and preferentially incorporated into PL. In addition, based on the rapid increase followed by a decline in unesterified [^{14}C]MA with its minimal incorporation into TG and PL (Fig. 3), MA is more readily oxidized, but poorly utilized for lipid synthesis in the liver. LA is better incorporated into TG than into PL (Fig. 3), which may be stimulatory to hepatic VLDL synthesis. Therefore, its hypocholesterolemic effect may be related not to an inhibition of VLDL production (29,30), but to an increase in the rate of VLDL catabolism (19,20,31,32).

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Effect of Regulating Cholesterol Biosynthesis on Breath Isoprene Excretion in Men

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Isoprene is a normal constituent of human breath and may be derived from the cholesterol synthetic pathway. Acute and chronic lovastatin and a cholesterol-supplemented diet were used to determine whether a mechanistic link exists between isoprene and cholesterol biosynthesis *in vivo* in humans. The acute effects of lovastatin, a competitive inhibitor of the rate-limiting step of cholesterol biosynthesis, on breath isoprene excretion was determined by administering a single 20, 40 or 80 mg dose of this drug to five healthy male subjects at 8 p.m. and measuring their breath isoprene levels every 4 h for one 24 h cycle before and after treatment. When compared to the baseline cycle, all three doses of lovastatin significantly reduced breath isoprene levels at 6 and 10 h post-drug treatment. Chronic lovastatin therapy (40 mg b.i.d. for 6 wk) reduced 6 a.m. breath isoprene levels (time of maximum baseline value) by 27 ± 9% (SEM) and cholesterol synthesis measured in freshly isolated mononuclear leukocytes (ML) by 12 ± 6%. A cholesterol-supplemented diet (1070 mg, total) ingested for 6 wk reduced breath isoprene excretion and ML sterol synthesis by 16 ± 5 and 19 ± 4%, respectively. The parallel decreases in isoprene excretion and cholesterol synthesis caused by these pharmacologic and dietary means suggest that breath isoprene is derived from the cholesterol synthesis pathway.

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Hypercholesterolemia continues to be a major health problem. The majority of serum cholesterol is derived from *de novo* synthesis (1). The rate-limiting step of cholesterol synthesis is catalyzed by 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which reduces HMG-CoA to mevalonate and CoA. With the advent of a new class of compounds aimed at decreasing serum cholesterol by inhibiting HMG-CoA reductase activity, renewed interest in monitoring cholesterol synthesis *in vivo* in response to dietary and pharmacologic treatment has emerged.

The classical clinical procedure for assessing sterol synthesis is the sterol-balance method, where dietary intake of cholesterol is subtracted from fecal excretion of acidic and neutral sterols (2). Although this method continues to be used, it is cumbersome, cannot measure short-term changes in sterol synthesis, and is only valid when the patient is in a steady state. These disadvantages have limited the use of the sterol-balance method and, in response, other more indirect methods of assessing changes in cholesterol synthesis have been developed. These include measurements of serum mevalonate levels (3), quantitation of other cholesterol precursors in serum (4) and measurement of cholesterol synthesis or HMG-CoA reductase activity in freshly isolated mononuclear leukocytes (ML; Refs. 5–10).

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Abbreviations: DMPP, dimethylallyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; ML, mononuclear leukocytes.

Isoprene (2-methyl-1,3-butadiene) is a major component of human breath (11). The production of isoprene from mevalonate has been demonstrated in rat liver cytosol, and isoprene is thought to be a normal by-product of cholesterol synthesis formed by the nonenzymatic degradation of dimethylallyl pyrophosphate (DMPP; Refs. 12,13). Although measurable isoprene levels have not been found in the expired air of rats and several other animal species, isoprene has been identified in blood from these species by mass spectrometry (14).

The exact origin of isoprene found in human breath is not known. Cholesterol synthesis in humans appears to undergo a circadian rhythm with the peak at 6 a.m. (15). One of our laboratories has described a circadian rhythm for isoprene excretion with the peak between 2–6 a.m. (11). These results suggest that isoprene might also be produced from mevalonate as a by-product of cholesterol biosynthesis in humans. Therefore, a change in the rate of sterol synthesis might cause a comparable change in the amount of isoprene excreted. To investigate this possibility, we manipulated the rate of cholesterol biosynthesis of healthy volunteers using lovastatin or a cholesterol-enriched diet and compared breath isoprene excretion with their rate of cholesterol synthesis in isolated ML.

MATERIALS AND METHODS

Materials. Isoprene was purchased from Aldrich (Milwaukee, WI) and [^{14}C]acetate from ICN Radiochemicals (Irvine, CA). Other chemicals and reagents were purchased from common commercial sources.

Subjects. The acute studies with lovastatin were conducted in five healthy male laboratory personnel ranging in age from 18 to 50 years. For the chronic studies, eight healthy male subjects without significant heart or liver disease were recruited from the patient population of the Minneapolis VA Medical Center. The subjects ranged in age from 30–72 years, and their baseline serum cholesterol ranged from 189–318 mg/dL. All unnecessary medications were discontinued prior to the study, and necessary medications were continued throughout the study period.

In the chronic studies, all subjects ate meals prepared by the metabolic kitchen at the Minneapolis VA Medical Center consisting of 35% fat, 45% carbohydrates and 20% protein, with a cholesterol intake of 250 mg/d. For the cholesterol-supplemented diet, five eggs were isocalorically substituted so that the cholesterol-enriched diet contained 1070 mg total cholesterol per day. Weight was monitored at weekly intervals and calories readjusted to maintain a constant weight. These studies were approved by the Human Studies Committee of the Minneapolis VA Medical Center, and informed consent was obtained from all subjects.

ML harvest and sterol synthesis. ML were harvested from heparinized blood by gradient centrifugation and sterol synthesis quantitated in freshly isolated ML as previously described (5). Briefly, this consisted of measuring

the incorporation of [^{14}C]acetate (4.46 mmol, 2.24 mCi/mmol) into ML sterols by incubating 10^7 cells for 2 h in 5 mL of autologous serum.

Breath isoprene excretion. End expiratory alveolar breath samples were collected by having subjects exhale through a polyethylene tube into a glass collecting vial which allows all except the last 20 mL of breath to be vented. The vials were immediately capped and placed on dry ice until analyzed. Samples were collected in triplicate over a 3-min period and analyzed using a Perkin-Elmer (Norwalk, CT) sigma 2000 gas chromatograph equipped with an HS-100 headspace autosampler and a flame-ionization detector. Isoprene standards were prepared by volatilizing a 10-μL aliquot of pure isoprene in a 1.2-L gas septum bottle containing dry nitrogen gas. Serial dilutions of the latter were prepared using gas-tight syringes for the construction of a standard curve. Room air blanks and triplicate 31.6 nM isoprene gas standards were included in routine isoprene breath analyses. Prior to analysis, the samples were equilibrated for 10 min at 55°C in the heating compartment of the autosampler. Other gas chromatographic parameters were: column, 1/8 in. × 6 ft stainless steel; packing, Porapak Q (Alltech, Deerfield, IL); oven temperature, 150°C; carrier gas, nitrogen; carrier gas flow rate, 40 mL/min. The retention times for acetone and isoprene, the two most prominent peaks in breath, were 3.6 and 5.1 min, respectively. Breath isoprene values were calculated from the integrated peak areas of the breath samples and isoprene standards and expressed in units of nmol/L.

The inter- and intra-assay variability for the 31.6 nM isoprene standard based on peak area integration units from ten consecutive experiments and expressed as coefficient of variation were 3.1 and 2.3% respectively. The 10:00 a.m. breath isoprene for one subject determined over a period of 1 yr was 11.1 ± 1.4 nM (SD; n = 10), and the mean coefficient of variation for triplicate determinations of these same samples was 10.7%.

Statistical analysis. Statistical significance was determined by comparing each subject's value in response to a treatment to his baseline value using a paired *t*-test. All results are presented as the mean ± SEM.

RESULTS

Effect of lovastatin on breath isoprene excretion. We have previously demonstrated that as early as three h after an acute 80-mg dose of lovastatin, cholesterol synthesis (measured in freshly harvested ML) is inhibited by 57% (5). Therefore, we administered a graded dose of lovastatin to determine what effect the inhibition of cholesterol synthesis has on breath isoprene excretion. Breath isoprene showed a circadian rhythm with the peak between 2 and 6 a.m. in all five subjects tested (Fig. 1). The three doses of lovastatin administered at 8 p.m. (about 9 h prior to peak isoprene) caused a significant reduction in breath isoprene excretion at 2 and 6 a.m. of ⩾ 36% as compared to baseline values.

Chronic administration of lovastatin (40 mg b.i.d.) for 6 wk resulted in a modest 12% decrease in ML sterol synthesis (Table 1), a value similar to that previously reported (5), whereas breath isoprene was reduced by 26% in these subjects (Table 1). Their low-density lipoprotein and total cholesterol were reduced 41 and 32%, respectively (Table 1).

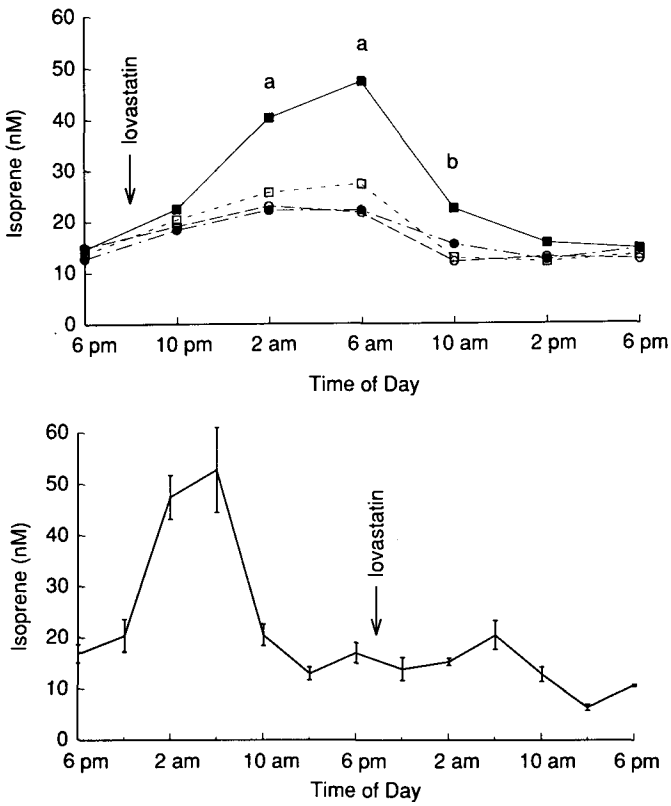

FIG. 1. Effect of acute lovastatin administration on breath isoprene excretion of five male subjects (upper panel); and a 24-h isoprene excretion cycle before and after lovastatin (80 mg) administration in a single subject (lower panel). Breath isoprene excretion was measured every four hours in five male subjects at baseline (■) and after the administration of a 20 mg (□), 40 mg (○) or 80 mg (●) dose of lovastatin. The doses were administered at 8 p.m., as shown. a, Different from baseline at all doses, $P < 0.05$; b, different from baseline for the 20 and 80 mg dose only, $P < 0.05$.

Effect of cholesterol feeding on breath isoprene excretion. Cholesterol feeding results in a decreased total body cholesterol synthesis due to feedback inhibition of HMG-CoA reductase (16,17). If isoprene is formed as a by-product of the cholesterol synthetic pathway, a decrease in sterol synthesis should reduce breath isoprene excretion. A high cholesterol diet (average 1070 mg/d) ingested for 6 wk reduced ML sterol synthesis 19% as compared to the rate of ML sterol synthesis measured on a low cholesterol diet (baseline; Table 1). A similar 16% reduction in breath isoprene excretion was observed at the end of the six-week period of high cholesterol intake (Table 1). However, the effect of increasing cholesterol intake on breath isoprene excretion could be appreciated as early as the first day, as evidenced by a 35 and 53% decrease in 6 a.m. breath isoprene levels of two subjects one day after cholesterol supplementation.

DISCUSSION

Isoprene is a normal occurrence, as well as the major hydrocarbon found in human breath, although its precise metabolic origin has not been established. Earlier reports

TABLE 1

Effect of Chronic Lovastatin and Cholesterol Feeding on Breath Isoprene Excretion and Mononuclear Leukocytes (ML) Sterol Synthesis[a]

Treatment	Breath isoprene (nM/L)	ML sterol synthesis (pmol/10^6/min)	LDL cholesterol (mg/dL)	Total serum cholesterol (mg/dL)
None (baseline)	21.7 ± 6.4	11.3 ± 0.8	122 ± 14	203 ± 6
Lovastatin	16.0 ± 4.5[b]	9.9 ± 0.7[b]	71 ± 10[c]	142 ± 11[d]
	(74 ± 9)	(88 ± 6)	(59 ± 5)	(68 ± 3)
Cholesterol	18.3 ± 5.5[b]	9.2 ± 0.8[b]	134 ± 10	212 ± 12
	(84 ± 5)	(81 ± 4)	(117 ± 13)	(103 ± 3)

[a]Breath isoprene and ML sterol synthesis were measured before (baseline) and after six weeks of lovastatin (40 mg b.i.d.) or a cholesterol-supplemented diet. The breath and blood samples were collected from male subjects between 6:00 and 7:00 a.m. Breath isoprene was measured in only six of the eight subjects studied. Data in parentheses are expressed as percent of baseline values. LDL, low-density lipoprotein.
[b]$P < 0.05$ relative to baseline.
[c]$P < 0.01$ relative to baseline.
[d]$P < 0.001$ relative to baseline.

have suggested that the amount of isoprene eliminated *via* the breath is not influenced by diet, age, sex or fasting (18), but rather is influenced by sleep patterns (19). We took advantage of the hypocholesterolemic agent, lovastatin, to test if isoprene originated from the cholesterol biosynthetic pathway *in vivo*. Our results demonstrated that acutely administered lovastatin blunted the early morning rise in breath isoprene excretion (Fig. 1), using doses of this drug that are known to inhibit sterol biosynthesis (5). Chronic lovastatin administration resulted in a small, but significant, decrease in isoprene excretion which agreed well with the decrease in sterol synthesis measured in ML (Table 1). The smaller effect of lovastatin on breath isoprene in the chronic situation is likely due to induction of HMG-CoA reductase by the competitive inhibitor, lovastatin (5). This enzyme induction tends to return cholesterol synthesis toward the pre-lovastatin baseline value.

In contrast to a pevious report suggesting that the dietary intake does not influence breath isoprene excretion (18), we found that a cholesterol-supplemented diet given for six weeks modestly reduced isoprene excretion (Table 1). The mechanism of this effect likely relates to the concurrent decrease in cholesterol synthesis (measured in ML) that occurs by feedback inhibition (16,17). Therefore, whereas most dietary changes likely will not influence breath isoprene excretion, those dietary manipulations that change total body cholesterol synthesis may also alter breath isoprene excretion.

The rate limiting step of sterol synthesis is catalyzed by HMG-CoA reductase and the products of this reaction are mevalonate, CoA and $NADP^+$. Mevalonate is then converted in the cytosol to isopentenyl pyrophosphate, which undergoes isomerization to DMPP (20). Upon acidification, rat liver cytosolic DMPP is rapidly converted to isoprene *via* an acid-catalyzed elimination reaction, which proceeds through a carbonium ion intermediate (12,13). However, it is not clear whether this nonenzymatic reaction accounts for isoprene formation under physiologic conditions. Certain plants also produce isoprene from DMPP. In these plants, this reaction is catalyzed by an Mg^{2+}-dependent enzyme (21). A similar, but still unidentified, enzyme may also be responsible for the conversion of DMPP to isoprene in mammalian tissue. A possible enzyme candidate for catalyzing isoprene formation is the Mg^{2+}-dependent isopentenyl pyrophosphate isomerase, which catalyzes the interconversion of isopentenyl pyrophosphate and DMPP. This isomerization reaction proceeds through the same carbonium ion intermediate (22) as occurs in the acid-catalyzed nonenzymatic conversion of DMPP to isoprene (13). A loss of a single proton from this carbonium ion would result in the formation of isoprene.

In conclusion, the parallel decrease in isoprene secretion and sterol synthesis in ML caused by feeding a cholesterol-supplemented diet or by acute or chronic lovastatin administration suggests that breath isoprene is derived from the cholesterol synthesis pathway in humans *in vivo*.

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Bile Acid and Very Low Density Lipoprotein Production by Cultured Hepatocytes from Hypo- or Hyperresponsive Rabbits Fed Cholesterol

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Two groups of rabbits, either hyperresponsive or hyporesponsive to dietary cholesterol, were selected after ten weeks of cholesterol feeding (0.2 g cholesterol/kg body weight per day). Bile acids and very low density lipoprotein (VLDL) production were determined in primary hepatocyte cultures from control, hyper- and hyporesponsive rabbits. Free cholesterol and cholesteryl ester contents in hepatocytes of the hyperresponsive rabbits was significantly increased. In contrast, lipid composition in hepatocytes of the hyporesponders was similar to that of control cells. Cholic acid was the predominant bile acid in the culture medium of hepatocytes together with small amounts of chenodeoxycholic and deoxycholic acids. The rate of cholic acid production by hepatocytes in the hyporesponsive group was two times higher than that in the hyperresponsive group. Bile acid production by control hepatocytes was slightly higher than in the hyperresponsive group. In contrast, secretion of VLDL cholesteryl ester was significantly increased by hepatocytes of the hyperresponsive rabbits. Similar differences in bile acid production were found between hypo- and hyperresponsive rabbits selected after five days of cholesterol feeding and subsequent maintenance on a low cholesterol diet for a period of one month. The results suggest that the increased rate of bile acid production could contribute to the apparent resistance of hyporesponders to the atherogenic diet.

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The inconsistent response of plasma cholesterol levels to increased dietary cholesterol intake has been described in rabbits, in various other animal species and in humans (1–7). Upon cholesterol feeding, plasma cholesterol concentrations usually vary from severe hypercholesterolemia in most animals (hyperresponders) to virtually unaltered cholesterol level in a small fraction of animals (hyporesponders). Hyperresponsiveness is characterized by accumulation of lipoprotein particles enriched in cholesteryl esters (8–10). The actual mechanism(s) of hyper- or hyporesponsiveness in various species, including humans, is not clear. Hyporesponsiveness to cholesterol ingestion could be due to: (i) low intestinal absorption of cholesterol; (ii) effective suppression of endogenous cholesterol biosynthesis; (iii) increased secretion of bile cholesterol; and (iv) a high rate of cholesterol oxidation to bile acids. The results of several recent studies allow one to suggest that an enhanced rate of cholesterol conversion to bile acids in the liver is a factor contributing to the hyporesponsiveness to a dietary cholesterol challenge (4–7). However, direct measurements on bile acid production in hepatocytes from hyper- and hyporesponders have not been reported. There are no data on altered lipoprotein production by the liver in hyper- and hyporesponsiveness.

Recent studies have shown that primary cultures of hepatocytes retain the ability to synthesize, metabolize and secrete bile acids (11–14). In addition, the hepatocyte culture model possesses the capacity to express *in vitro* the changes in bile acid secretion that have been induced *in vivo* (13,14). We used primary cultures of hepatocytes from rabbits that were hyper- or hyporesponsive to dietary cholesterol to determine whether this responsiveness is related to the rates of bile acid and very low density lipoprotein (VLDL) production.

MATERIALS AND METHODS

Materials. All chemicals used were reagent grade. Minimal essential medium (MEM) with Earle's salts, fetal bovine serum (FBS), MEM nonessential amino acids, kanamycin, L-glutamine, and 150-mm and 100-mm culture dishes were purchased from Flow Laboratories, Inc. (Zwanenburg, The Netherlands). Collagenase 1200 units/mg and trypsin inhibitor were products of Sigma Chemical Co. (St. Louis, MO).

Animals. Male rabbits of the Chincilla breed, weighing 2.5–3 kg, were used. All rabbits were kept individually in stainless steel cages with wire-mesh bases. In the first series of experiments, control animals were maintained on a standard laboratory rabbit chow and, in addition, received vegetables. The experimental group (44 animals) received crystalline cholesterol mixed with vegetables (0.2 g/kg body weight per day). Vegetables were shredded and thoroughly mixed with crystalline cholesterol (6 g cholesterol/kg vegetable). Each animal received and ate 80–100 g of the mixture, according to body weight. Groups of rabbits hyperresponsive or hyporesponsive to dietary cholesterol were selected from the upper and lower sextiles of the plasma cholesterol concentrations after ten weeks of cholesterol feeding. Six animals from each group were used for the preparation of hepatocyte cultures. Blood samples were taken from the ear vein before killing, and samples of bile were collected from the gallbladder immediately after killing the animals. All samples were taken at the same time of the diurnal cycle (between 11 a.m. and 12 a.m.). In a second series of experiments, the group of rabbits (30 animals) with plasma cholesterol levels ranging from 19.3 to 235.7 mg/dL (average level, 66.1 ± 7.9 mg/dL) received standard laboratory chow and crystalline cholesterol mixed together with vegetables (0.2 g/kg body weight per day). After five days of feeding, blood samples were taken from the ear vein and the five

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Abbreviations: B/E, apoprotein B and apoprotein E; FBS, fetal bovine serum; GLC, gas–liquid chromatography; HPTLC, high-performance thin-layer chromatography; MEM, Eagle's minimum essential medium; VLDL, very low density lipoprotein.

rabbits with the lowest plasma cholesterol concentration were designated as hyporesponders, and the five rabbits with the highest plasma cholesterol concentration were designated as hyperresponders. Then cholesterol was omitted from the diet, and both groups of rabbits received standard laboratory chow for one month. After this period, the plasma cholesterol levels in both groups were within control limits. Three animals from each group were killed and used for the preparation of primary hepatocyte cultures.

Preparation and culture of hepatocytes. Rabbit liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described in detail elsewhere (15). The cells were plated in 100-mm dishes (3 or 6 dishes per animal) at a density of 2×10^5 cells/cm^2 and cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, kanamycin (100 μg/mL) and nonessential amino acids (1%) at 38°C in a 95% air/5% CO_2 atmosphere. The characteristics of primary cultures of rabbit hepatocytes have been described earlier (16). Cells were incubated for 24 h in MEM supplemented with 10% FBS, then for 24 h in MEM supplemented with 10% of control rabbit serum and then for 24 h in MEM without serum. Samples of the serum-free medium were analyzed.

Analytical methods. Bile acids in the culture medium and bile were quantitated with gas–liquid chromatography (GLC) using a 50-m SE-30 capillary column (Alltech Associates, Inc., Deerfield, IL) (17–19). Bile acids used as a standard for GLC were purchased from Calbiochem (La Jolla, CA). Allodeoxycholic acid was synthesized as described previously (20), and purified by preparative thin-layer chromatography. All bile acids were at least 98% pure as judged by GLC. Conjugated bile acids were extracted after isolation of a total lipid fraction (21) using a reverse-phase cartridge (Sepak C-18, Waters Associates, Milford, MA) according to Kempen *et al.* (22) and were separated by high-performance thin-layer chromatography (HPTLC) using chloroform/methanol/glacial acetic acid/water (15:5:1:1, by vol) as developing system. Densitometry (564) nm) on HPTLC plates was carried out after treatment with 10% phosphomolybdenic acid and subsequent bleaching of the background in ammonia vapor. Taurocholate, taurodeoxycholate and glycocholate were used as references. VLDL fractions secreted by the cells were isolated by preparative ultracentrifugation of the medium using an SW-55 rotor (Beckman Instruments, Palo Alto, CA) at 45,000 rpm for 18 h at 10°C. VLDL were collected from the centrifugation tubes at d < 1.006 g/mL (1 mL from the top). Neutral lipids of VLDL, plasma and cells were extracted according to Goldstein *et al.* (23), and individual lipid fractions were separated by HPTLC (silica gel plates; Merck, Darmstadt, Germany) with hexane/diethyl ether/acetic acid (70:30:2, by vol) as developing system. Triacylglycerols, free cholesterol and cholesteryl esters were quantitated by densitometry on the HPTLC plates (24,25) using an automated Camag HPTLC/TLC scanner (Muttens, Switzerland) connected to a recording integrator SP 4100 (Spectra Physics, Darmstadt, Germany). Each determination was compared with standard run using known amounts of cholesterol, triolein and cholesteryl oleate.

Plasma total cholesterol for selection of experimental groups was assayed enzymatically using the Total Cholesterol Kit (Medix, Helsinki, Finland) and the Chemistry Analyzer FP-901 (Labsystems, Oy, Finland). Ten μL of plasma or standard solution was combined with 1 mL of cholesterol enzymatic reagent and incubated at 37°C for 20 min. Absorbance at 500 nm was measured for each sample against a reagent blank.

Statistical analysis. Results were analyzed by Student's *t*-test and expressed as means ± SE of the mean. Values of $P < 0.05$ were considered significant.

RESULTS

Analysis of plasma samples by GLC showed that after 10 wk of cholesterol feeding mean cholesterol concentrations in the hyperresponsive group were 17 times higher than in the control group, whereas the hyporesponsive rabbits showed only insignificant increase in cholesterol levels (Table 1). Esterification of plasma cholesterol was significantly increased in the hyperresponsive rabbits, which is typical for alimentary hypercholesterolemia in rabbit (26).

The bile acid composition of rabbit gallbladder bile is shown in Table 2. Secondary bile acids, *i.e.,* deoxycholic acid, allodeoxycholic acid and a small amount of lithocholic acid, were the predominant biliary bile acids. Primary bile acids, *i.e.,* cholic acid and traces of chenodeoxycholic acid, were also present. The mean biliary bile acid concentrations were similar in all groups. Yet, there was a tendency toward an increase in cholic acid concentration in the hyporesponsive group.

There was no significant difference in plating efficiency of hepatocytes obtained from animals of the three experimental groups. The viability of hepatocyte monolayers after 24 h in culture was determined as described earlier (27), and was greater than 90%. The free cholesterol and, particularly, the cholesteryl ester contents of hepatocytes

TABLE 1

Serum Cholesterol Concentration of Rabbits Fed the Control Diet and Hypo- and Hyperresponsive Rabbits Fed the Cholesterol-Rich Diet for Ten Weeks[a]

	Control	Hyporesponsive	Hyperresponsive
Cholesterol total[b]	61 ± 3	108 ± 30	912 ± 65[c,d]
Cholesteryl esters[c]	32 ± 3	40 ± 5	64 ± 2[c,d]

[a]Values are means ± SE for six animals in each group.
[b]mg Cholesterol/dL of plasma.
[c]$P < 0.05$, *vs.* control group.
[d]$P < 0.05$, *vs.* hyporesponsive group.

TABLE 2

Biliary Bile Acid Composition of Rabbits Fed the Control Diet and Hypo- and Hyperresponsive Rabbits Fed the Cholesterol-Rich Diet for Ten Weeks[a]

Acid[b]	Control	Hyporesponsive	Hyperresponsive
Lithocholic	2.4 ± 0.3	3.0 ± 0.7	3.4 ± 0.9
Allodeoxycholic	12.5 ± 1.0	12.1 ± 2.6	10.2 ± 2.2
Deoxycholic	108.8 ± 4.1	104.5 ± 3.6	94.0 ± 13.1
Cholic	3.9 ± 0.5	7.1 ± 1.6	4.8 ± 1.2

[a]Values are means ± SE for six animals in each group.
[b]mg of Acid/mL of bile.

of the hyperresponsive animals were significantly higher (by 26 and 102%, respectively) than those of control cells (Table 3). In contrast, only cholesteryl ester content was slightly increased in cells of hyporesponders. It is noteworthy that the triacylglycerol content was decreased in the hyperresponsive group.

In agreement with previous studies (11), cholic acid was the major primary bile acid in the hepatocytes culture medium, while the amount of chenodeoxycholic acid was small. In addition, the presence of traces of secondary bile acids, deoxycholic, lithocholic and ursodeoxycholic acids could be detected. Figure 1 shows the time-course of primary bile acid accumulation in medium and cells in rabbit primary monolayer hepatocyte cultures over a period of 30 h. The cholic acid and chenodeoxycholic acid concentrations in the medium increased at a constant rate for 30 h, and only 1-5% of the amount was associated with the hepatocytes during this period. The data demonstrate that under our experimental conditions secretion of *de novo* synthesized primary bile acids does occur, that secretion of bile acids previously absorbed from the serum is very small and that bile acid content in culture medium mainly reflects bile acid biosynthesis by the hepatocytes.

Cholic acid production comprised more than 90% of total bile acid production in each group. After ten weeks of cholesterol feeding, the production of cholic acid by hepatocytes from the hyporesponsive rabbits was twofold higher than that from the hyperresponsive animals (Table 4), while the values for the latter group were within the control limits. In contrast to cholic acid, secretion of VLDL cholesteryl esters by hepatocytes was increased in the hyperresponsive group. In the hyporesponsive group, secretion of VLDL cholesteryl ester was only moderately enhanced and was accompanied by a proportional increase in VLDL triacylglycerol secretion. Secretion of free cholesterol tended to increase in both cholesterol-fed groups.

To test the hypothesis that observed differences in bile acid production between hypo- and hyperresponsive animals were induced by prolonged cholesterol intake, groups of hypo- and hyperresponsive rabbits were selected after five days of cholesterol feeding (see Materials and Methods). After these five days the plasma cholesterol level of the hyporesponsive rabbits was essentially unchanged, whereas in hyperresponsive rabbits it was increased 2.1-fold (Table 5). Hepatocytes were isolated from the liver of hypo- and hyperresponders after one month of feeding the low cholesterol diet. Bile acid production

TABLE 3

Lipid Composition of Hepatocytes from Rabbits Fed the Control Diet and Hypo- and Hyperresponsive Rabbits Fed the Cholesterol-Rich Diet for Ten Weeks[a]

Lipid[b]	Control	Hyporesponsive	Hyperresponsive
Free cholesterol	35.9 ± 3.6	37.6 ± 1.3	45.3 ± 0.8[c,d]
Cholesteryl esters	15.1 ± 0.8	19.4 ± 1.6[c]	30.5 ± 3.3[c,d]
Triacylglycerols	30.6 ± 4.0	27.9 ± 3.6	19.6 ± 2.3[c]

[a]Values are means ± SE for five animals in each group.
[b]μg of Lipid/mg of cell protein.
[c]$P < 0.05$, *vs.* control group.
[d]$P < 0.05$, *vs.* hyporesponsive group.

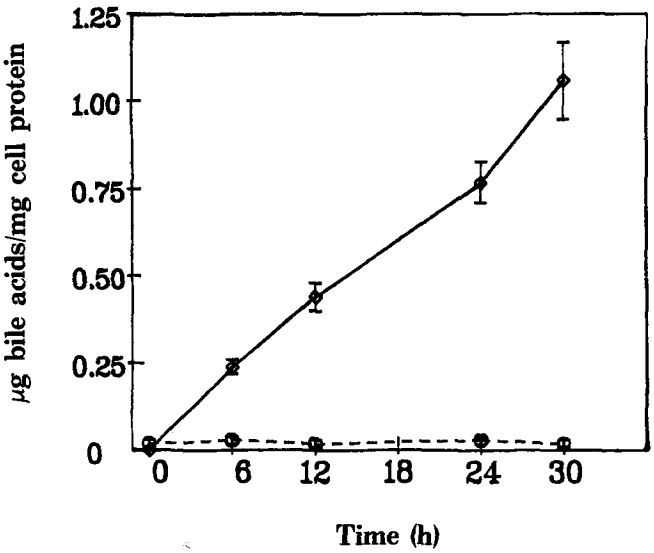

FIG. 1. Time-course of primary bile acids accumulation in culture medium and cells in primary rabbit hepatocyte cultures. Hepatocytes were harvested at the time indicated, frozen-thawed and homogenized, and bile acid content was determined in cells and medium by capillary gas-liquid chromatography. Results are expressed as means ± SE for three plates. Solid line, bile acids accumulation in medium; dashed line, bile acid accumulation in hepatocytes.

TABLE 4

Cholic Acid and VLDL Lipid Secretion by Hepatocytes from Rabbits Fed the Control Diet and from Hypo- and Hyperresponsive Rabbits Fed the Cholesterol-Rich Diet for Ten Weeks[a]

Lipid[b]	Control	Hyporesponsive	Hyperresponsive
Cholic acid	0.61 ± 0.12	0.98 ± 0.10[c]	0.47 ± 0.05[d]
Free cholesterol	2.02 ± 0.26	2.32 ± 0.31	2.41 ± 0.18
Cholesteryl esters	0.38 ± 0.01	0.57 ± 0.07[c]	1.42 ± 0.37[c,d]
Triacylglycerols	3.04 ± 0.20	4.42 ± 0.30[c]	2.98 ± 0.10[d]

[a]Values are means ± SE. Cholic acid (n = 6) and n = 5 for neutral lipids.
[b]μg of Lipid/mg of cell protein/24 h.
[c]$P < 0.05$, *vs.* control group.
[d]$P < 0.05$, *vs.* hyporesponsive group.

TABLE 5

Plasma Cholesterol Levels of Hypo- and Hyperresponsive Rabbits Selected After Five Days of Cholesterol Feeding at Different Stages of Feeding[a,b]

Stage of feeding	Hyporesponsive	Hyperresponsive
Before cholesterol feeding	42.3 ± 10.6	114.5 ± 36.1
After five days of feeding with cholesterol-rich diet	50.8 ± 3.4	242.6 ± 42.3[c]
After one month of feeding with low cholesterol diet	38.4 ± 14.4	140.4 ± 20.4[c]

[a]Values are means ± SE for five animals in each group.
[b]mg Cholesterol/dL of plasma.
[c]$P < 0.05$, *vs.* hyporesponsive group.

TABLE 6

Secretion of Cholic Acid Conjugates by Hepatocytes from Hypo- and Hyperresponsive Rabbits Selected After Five Days of Cholesterol Feeding and Subsequent One-Month Feeding a Standard Diet[a]

Acid[b]	Hyporesponsive	Hyperresponsive
Taurocholic acid	0.87 ± 0.04	0.56 ± 0.07[c]
Glycocholic acid	0.28 ± 0.04	0.14 ± 0.02[c]
Taurocholic/glycocholic ratio	3.56 ± 0.90	4.27 ± 0.89

[a]Values are means ± SE for three animals in each group.
[b]μg of Acid/mg of cell protein/24 h.
[c]$P < 0.05$, *vs.* hyporesponsive group.

by primary hepatocytes was measured by HPTLC to determine whether there was any difference between these two groups in the production of glyco- and taurocholic conjugates. Table 6 shows that the production of both taurocholic and glycocholic acids was significantly increased in the hyporesponsive group as compared to the hyperresponsive group (1.5- and 2-fold, respectively). The difference in taurocholic/glycocholic acid ratio was not statistically significant. The results suggest that differences in the rate of bile acid production are not due to prolonged cholesterol intake.

DISCUSSION

The mechanisms responsible for the large differences in the response of plasma cholesterol levels to dietary cholesterol intake have been investigated in many animal and human studies (1-7, 28-30). It has been shown that there is a small but significant increase in cholesterol absorption efficiency in hyperresponsive rabbits as compared to hyporesponsive ones (5,6). In addition, in some human populations cholesterol absorption efficiency was shown to correlate with plasma cholesterol levels (31). At the same time it was found that in low-responders, cholesterol feeding significantly increased fecal excretion of bile acids. In hyperresponders this increase was either not observed or was insignificant (4-6). These results suggest that effective oxidation of absorbed cholesterol to bile acids, as well as decreased cholesterol absorption, could explain resistance to increased dietary cholesterol intake.

The present study showed for the first time that primary hepatocytes of hyporesponsive rabbits produce significantly higher amounts of bile acids as compared to control and hyperresponsive animals. The observed tendency toward an increase in cholic acid concentration in the hyporesponsive group apparently reflects a higher rate of synthesis. However, we found that the concentrations of secondary bile acids were the same in the bile of hypo- and hyperresponsive rabbits. It seems possible that increased production of bile acids in hyporesponders is balanced by increased fecal loss. Recently it has been shown that fecal loss of bile acids is increased in hyporesponsive rabbits (5). The results of the second series of experiments demonstrate that the difference in bile acid production between hypo- and hyperresponsive animals is not induced by prolonged cholesterol feeding and suggest that the differences that are seen between the experimental groups of animals existed before the cholesterol challenge.

It is known that the rate of bile acid production is dependent upon the activity of 7α-hydroxylase and that enhanced production, as a rule, is the result of increased enzyme activity. It seems probable that the low level of bile acid production in hyperresponders may be related to the low activity of 7α-hydroxylase. This would agree with the finding that the activity of 7α-hydroxylase is considerably lower in hyperresponsive pigeons than hyporesponsive ones (32). The cause for the different activity of 7α-hydroxylase in hyper- and hyporesponsive animals remains unclear. It is generally assumed that the rate of bile acid synthesis is regulated by bile acids returning to the liver *via* the portal vein. Recently, in rats and rabbits it was found that different bile acids suppress bile acid synthesis and 7α-hydroxylase activity to different extents (33-35). Therefore, the activity of this enzyme and rate of bile acid production can be linked to the composition of the existing bile acid pool. However, the similar bile acid compositions of gallbladder bile in hypo- and hyperresponsive animals (Table 2) suggest that this explanation is not valid in the present case.

Liver cells from hypercholesterolemia-resistant rabbits appear to show an altered cholesterol metabolism, as compared to normal animals (36). Liver membranes prepared from resistant animals have increased activity of apoprotein B and apoprotein E (B/E) receptors and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and decreased acyl-coenzyme A:cholesterol acyltransferase activity both during cholesterol feeding and on a low-cholesterol diet. The resistant animals maintain a low intracellular cholesterol concentration, and it was suggested that the liver cells in these animals are able to degrade cholesterol by an undefined mechanism (36). Our data suggest that this mechanism is the oxidation of excess cholesterol to bile acids and that decreased bile acid production contributes to the accumulation of free and esterified cholesterol in hepatocytes of hyperresponsive rabbits. According to previous studies (8-10), an increase in the cellular cholesteryl ester pool was accompanied by stimulated secretion of cholesteryl ester in VLDL. While hepatocytes of hyporesponsive rabbits secreted moderately increased amounts of VLDL neutral lipids, hepatocytes of hyperresponsive rabbits secreted significantly increased amounts of total cholesterol and cholesteryl ester. Because cholesterol accumulation in cells inhibits the activity of B/E receptors, our data could explain the low B/E receptor activity found in the liver of hyperresponders (30,36). Decreased activity of B/E receptors and an increased rate of lipoprotein cholesterol secretion could account for the rapid accumulation of cholesterol in the blood of hyperresponsive rabbits.

The present studies demonstrate that the rate of bile acid production in hyperresponsive rabbits is significantly lower than in the hyporesponsive rabbits. Prolonged cholesterol feeding induces significant increase in secretion of VLDL free and esterified cholesterol in hyperresponders; in contrast, only a slight increase in secretion of VLDL cholesteryl esters is observed in hyporesponders. These data suggest that, in rabbits, a defect in the elimination of cholesterol from the body in the form of bile acids could contribute to the development of hypercholesterolemia.

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Mouse Alveolar Surfactant: Characterization of Subtypes Prepared by Differential Centrifugation

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To characterize the properties of alveolar surfactant subfractions obtained from mouse lung by differential centrifugation, lavage fluid, following a preliminary centrifugation at 140 × *g* for 5 min to yield a cellular pellet (Pc), was sequentially centrifuged at 10,000 × *g* for 30 min, 60,000 × *g* for 60 min and 100,000 × *g* for 15 h; and the resultant pellets, respectively referred to as P10, P60 and P100, were harvested for electron microscopy, phospholipid analysis and surface tension measurements. Ultrastructural differences were observed, in that P10 contained large multilamellated structures which were typical of newly secreted surfactant, P100 contained small unilamellar vesicular structures, typical of catabolic end products of alveolar surfactant and P60 appeared to contain a mixture of structures present in P10 and P100 in addition to numerous, large unilamellar vesicles which were not present in either P10 or P100. Slight but significant differences were found in the phospholipid compositions of the three subfractions but not in the fatty acid composition of their phosphatidylcholine (PC) component. There were no significant differences in their disaturated PC/total PC ratios, but significant differences in their phospholipid/protein ratios. P60 had the highest proportion of phospholipid to protein. P10 and P60 demonstrated surface activity but P100 did not. Total alveolar surfactant phospholipid was evenly distributed among the three fractions. This pattern of distribution was significantly different from that observed in rabbit subfractions prepared by the same procedure. These data indicate that mouse alveolar surfactant consists of three distinct subfractions or subtypes which can be separately and quantitatively isolated by differential centrifugation. They also suggest that there may be species differences in the relative proportions of the individual subtypes present in normal adult lung.

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Pulmonary surfactant is a phospholipid-rich material which lines the mammalian lung and functions to promote alveolar stability by reducing the surface tension at the air-alveolar interface (1,2). Surfactant is synthesized in the large alveolar Type II cell and stored in multilamellar intracellular vesicles (lamellar bodies) which are ultimately released to the alveolar space (3).

Recent evidence indicates that alveolar surfactant exists in at least three macromolecular forms, or subtypes, that can be separated on the basis of their sedimentation properties (4–9). Characterized according to their ultrastructure, surface properties and composition (see Refs. 10 and 11 for reviews), labelling studies have suggested that these subtypes may be in metabolic sequence such that the larger, heavier subtypes, which consist of multilamellated structures and tubular myelin figures, constitute a newly secreted form of alveolar surfactant, whereas the smaller, ligher subtypes, which consist of small unilamellar vesicular structures, appear to be catabolic end products of the secreted material and thus represent a used or "spent" form of alveolar surfactant. Current evidence suggests that this catabolic end product may ultimately be taken up and reutilized by the Type II cell for new lamellar body production (12–14).

In considering the entire "life cycle" of the pulmonary surfactant, the processes controlling synthesis, secretion and re-uptake have been the most thoroughly studied (11). Recent interest has focused on the intra-alveolar processing or metabolism of the secreted surfactant particularly, as it is becoming increasingly apparent that these processes may be drastically altered in certain pathological conditions involving lung injury (15–18). However, as the number of studies reported to date has been somewhat sparse (4,9,15–20), our current knowledge of this topic is still rather limited.

One part of the problem is the lack of uniformity among investigators in the preparation of the subfractions for analysis. In earlier studies, done primarily on rabbits (4,6) and rats (7), stepwise differential centrifugation schemes were used. Though there was some variation in the precise scheme used, there appeared to be species differences in the pattern of distribution of phospholipids over the subfractions. In the rabbit, for example, the bulk of the phospholipid was recovered in the pelleted fractions, whereas in the rat, large amounts of phospholipid were recovered in the final supernatant fraction. This suggests the possibility of species variation in the relative rates of subtype conversion and/or uptake, an important point to consider in attempting to understand the entire surfactant "life cycle" not only in the normal lung but in the diseased lung as well.

The mouse provides a useful model for studying alveolar injury, having been used not only by ourselves (21) but by several other investigators as well (8,9,13,15). Though this model has been extensively studied by Gross and co-workers (8,9,14,15,16), this group did not use a differential centrifugation scheme to isolate the alveolar subfractions, but rather a somewhat more elaborate density gradient technique which requires the dropwise collection of fractions for characterization and analysis. This is not only time-consuming and cumbersome, but it also makes interspecies comparisons difficult. It would therefore be of value to develop a more convenient and equally consistent and reliable technique for isolating the individual subfractions in this model. The objective of this study was therefore to employ a differential centrifugation scheme to prepare alveolar surfactant subfractions from normal mouse lung, characterize the properties of the individual fractions and, where pertinent, make comparisons with fractions similarly prepared from healthy, adult rabbit lung.

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Abbreviations: Pc, low-speed pellet; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SP-A, surfactant protein A; SP-D, surfactant protein D; TLC, thin-layer chromatography; X, unidentified phospholipid.

MATERIALS AND METHODS

Random-bred, pathogen-free, Carworth Farms White, male mice, 8–9-weeks-old, were used in the study. Following an intraperitoneal injection of sodium pentobarbital, the lungs were lavaged with 0.85% NaCl in 4 × 1.0 mL aliquots, as described previously (22).

The lavage washings from 2–5 mice were pooled and, following a 5 min centrifugation at 140 × *g* to remove a cellular pellet (Pc), surfactant subfractions were obtained by sequential centrifugation at 10,000 × *g* for 30 min, 60,000 × *g* for 60 min and 100,000 × *g* for 15 h. The pellet subfractions are referred to as P10 (10,000 × *g* pellet), P60 (60,000 × *g* pellet) and P100 (100,000 × *g* pellet), respectively. The final (100,000 × *g*) supernatant is referred to as S100.

Following the lavage procedure, the lungs were removed and pooled (as indicated above for the lavage) for isolation of the tissue-stored surfactant (lamellar bodies) using a procedure involving differential and density gradient centrifugation as previously described (23).

Aliquots of each lavage fraction and lamellar body preparation were extracted with chloroform/methanol (2:1, vol/vol) for phospholipid analysis as described previously (23). Individual phospholipids were separated by two-dimensional thin-layer chromatography (TLC) as described elsewhere (24). Further aliquots were removed for analysis of disaturated phosphatidylcholine (PC) according to the method of Mason *et al.* (25) as previously described (26). For fatty acid analysis of PC and phosphatidylglycerol (PG), these two phospholipid classes were scraped individually from the TLC plate following two-dimensional TLC as described (24), and methyl esters of their fatty acyl chains were obtained by transesterification with 10% BF_3 in methanol (wt/vol) and extraction with petroleum ether (27).

The methyl esters were analyzed by gas–liquid chromatography as described by Cook (28).

Aliquots of each surfactant fraction were removed for protein analysis according to the procedure of Lowry *et al.* (29). To prevent interference from the lipid constituents, each sample was extracted with an equal volume of chloroform before reading the absorbance as previously described (30). Surface activity was assessed using the pulsating bubble surfactometer as described by Enhorning (31). For these measurements the concentration of each preparation was adjusted so that it contained 5 mg phospholipid/mL. In some experiments samples were fixed in 0.1 M cacodylate buffered 2.5% glutaraldehyde (pH 7.3) and processed for electron microscopy as described previously (18).

For comparative purposes, several adult male New Zealand white rabbits (weighing 2.0–2.5 kg) were used to prepare alveolar surfactant subfractions and lamellar bodies. With the exception that 50-mL aliquots were used for the lavage procedure, all other procedures were as described above. Only phospholipid analyses were performed on these fractions.

Statistical comparison of the results was performed using Student's *t*-test (32) or Duncan's new multiple range test (33). For the Duncan's test, an analysis of variance was performed before the test. The F value was significant to 5%.

RESULTS

Electron microscopy of the individual lavage fractions obtained from mouse lung revealed ultrastructural differences in the material sedimenting under the different centrifugal conditions used in this study (Fig. 1). As

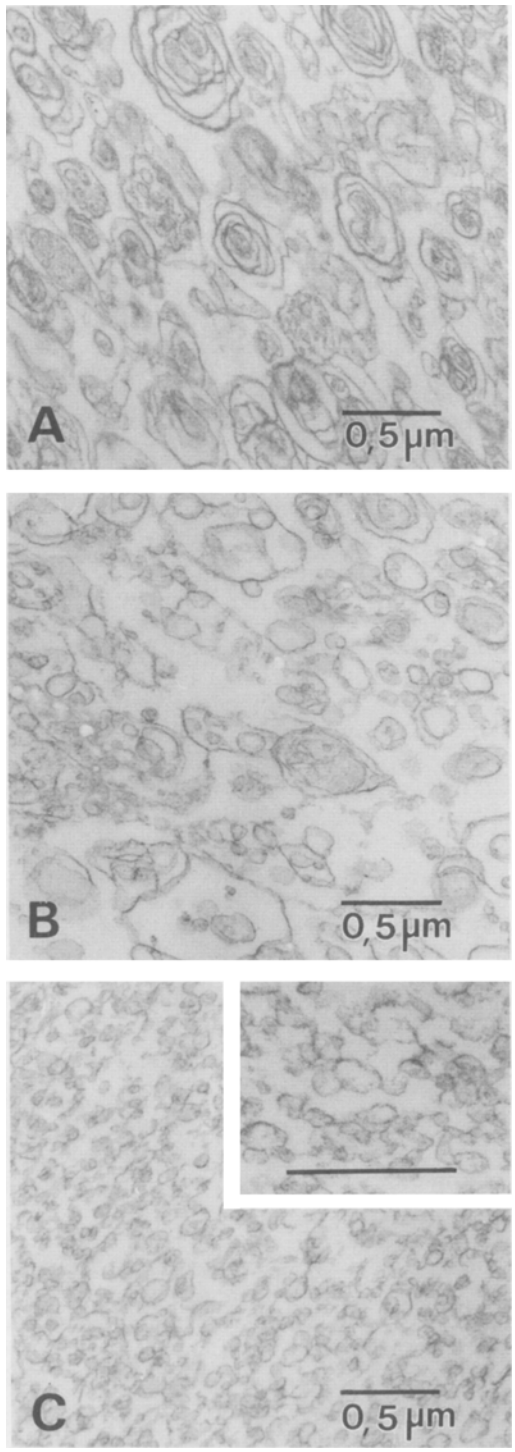

FIG. 1. Electron micrographs of mouse alveolar surfactant subfractions prepared as pellets by sequential centrifugation of lavage returns at (A): 10,000 × *g* for 30 min (P10); (B): 60,000 × *g* for 60 min (P60); and (C): 100,000 × *g* for 15 h (P100) following a preliminary centrifugation for 5 min at 140 × *g* to remove a cellular pellet. Magnification: A × 26,250; B × 26,250; C × 26,250 (inset × 48,750).

TABLE 1

Surface Activity of Mouse Surfactant Subfractions[a]

Fraction	n	γ Max.	γ Min.	Time to reach γ min. (s)
P10	4	37.4 ± 6.7	1.8 ± 3.4	0
P60	2	45.2[b]	7.4[c]	420
P100	3	54.3 ± 12.1	16.6 ± 3.4	317 ± 75
Lamellar bodies	5	37.7 ± 2.5	2.7 ± 1.7	10 ± 5

[a]Alveolar subfractions were prepared by sequential centrifugation of mouse lung lavage as described in the legend to Figure 1, and lamellar bodies by a procedure of differential and density gradient centrifugation as previously described (Ref. 23). Each sample contained 5.0 mg phospholipid/mL. γ, Surface tension (mN/m) as measured by pulsating bubble technique; γ max., surface tension at maximum bubble radius (0.55 mm); γ min., surface tension at minimum bubble radius (0.25 mm); each value represents the mean ± 1 SD for the number of determinations shown.
[b]Individual values: 45.8, 44.5.
[c]Individual values: 4.9, 9.8.

TABLE 2

Phospholipid/Protein Ratio of Mouse Alveolar Surfactant Subfractions[a]

Fraction	μmol Phospholipid/mg protein[b]
Lavage fluid	1.108 ± 0.299
Pc	0.536 ± 0.222
P10	3.205 ± 0.697[c]
P60	7.463 ± 1.509[c]
P100	4.403 ± 0.614[c]
S100	0.080 ± 0.062

[a]Fractions P10, P60 and P100 were prepared as described in the Legend to Figure 1. Pc represents the cellular pellet and S_{100} the final supernatant fraction obtained following the 15-h centrifugation at 100,000 × *g*.
[b]Each value represents the mean ± 1 SD for five determinations.
[c]Significantly different from unfractionated lavage fluid and each of the individual subfractions ($P < 0.01$ by Duncan's Multiple Range Test).

indicated, P10 (Fig. 1A), which represents the most easily sedimentable subfraction, consisted mainly of multilamellated structures typical of newly-released surfactant while P100 (Fig. 1C), which represents the least easily sedimentable subfraction, consisted of much smaller unilamellar vesicular structures typical of catabolic forms of P10. P60 was less easily defined. Although it appeared to contain structures which were present in both P10 and P100, it also contained many large unilamellar vesicles which were not found in either P10 or P100. Also of interest is that the membranes of the structures present in P100 were much more diffuse than those in either P10 or P60. Not enough material was present in Pc for electron microscopy.

Differences were also observed in the surface properties of the surfactant subfractions (Table 1). Of the alveolar subfractions only P10 and P60 could be regarded as being surface active, in that they both reduced the surface tension to less than 10 mN/m, with P10 being more surface active than P60 in that it reduced the surface tension to lower values and in less time. In this regard the surface activity profile of P10 was similar to that of the isolated lamellar body fractions.

The phospholipid/protein ratios of the individual pellet subfractions obtained from the mice were significantly different from each other (Table 2). Each was also significantly different from that of the unfractionated lavage fluid, the cellular pellet and the final resultant supernatant fraction (S100). Of all the pellet subfractions, P60 had the highest ratio of phospholipid to protein. There were small but significant differences in the phospholipid composition of the mouse alveolar surfactant subfractions (Table 3) in that P100 contained relatively more PC than P10 and P60 and slightly less PG and phosphatidylethanolamine (PE) than P10. In a previous report (18) we found that the cellular pellet contained somewhat less (approximately 70% of the total lipid phosphorus) PC than is reported here for the surfactant subfractions, as well as up to 5% lysophosphatidylcholine, a phospholipid not present in the surfactant subfractions.

No significant differences were found in the disaturated PC/total PC ratio of the individual surfactant subfractions obtained from mouse lung. These values were 0.569 ± 0.033, 0.589 ± 0.022, 0.566 ± 0.025 and 0.597 ± 0.027 (n = 6) for P10, P60, P100 and S100, respectively. Not enough material was available for analysis of Pc.

No significant differences were found in the PC fatty acyl composition of the alveolar surfactant subfractions (Table 4). Palmitic acid (16:0) was the most abundant fatty acid for all three subfractions. Although this fatty acid also constituted the most abundant species in the isolated lamellar bodies, it was slightly but significantly reduced in comparison to the alveolar subfractions. Oleic acid (18:1) was slightly higher in lamellar bodies than in the alveolar surfactant subfractions.

Only a few samples were available for PG fatty acyl analysis. As with PC, the most abundant fatty acid

TABLE 3

Phospholipid Composition of Mouse Alveolar Surfactant Subfractions[a]

Fraction	Number of determinations	Percent of total lipid phosphorus[b]						
		PC	PG	PI	PS	SM	PE	X
P10	5	81.2 ± 0.8	13.0 ± 0.4	1.8 ± 0.2	0.5 ± 0.1	0.2 ± 0.5	2.4 ± 0.5	0.9 ± 0.6
P60	3	81.8 ± 0.7	11.7 ± 1.2	1.8 ± 0.1	1.5 ± 1.2	0.4 ± 0.4	2.1 ± 0.4	0.6 ± 0.5
P100	5	83.8 ± 0.7[c]	11.4 ± 0.8[d]	1.7 ± 0.6	0.7 ± 0.5	0.2 ± 0.3	1.4 ± 0.4[d]	0.8 ± 0.7

[a]Fractions were prepared as pellets by sequential centrifugation of alveolar lavage returns as described in the legend to Figure 1.
[b]Each value represents the mean ± 1 SD for the number of determinations shown. Phospholipids include: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PE, phosphatidylethanolamine; X, unidentified phospholipid.
[c]Significantly different from P10 and P60 ($P < 0.05$ by Duncan's Multiple Range Test).
[d]Significantly different from P10 ($P < 0.05$ by Duncan's Multiple Range Test).

TABLE 4

Phosphatidylcholine Fatty Acyl Composition of Mouse Surfactant Subfractions[a]

Fatty acid	P10 (3)	P60 (3)	P100 (3)	Lamellar bodies (5)
14:0	3.3 ± 0.6	3.2 ± 0.3	3.4 ± 0.3	3.1 ± 0.7
16:0	76.7 ± 5.3	74.8 ± 4.4	73.8 ± 4.8	67.0 ± 4.9[b]
16:1	12.8 ± 5.9	14.6 ± 2.6	13.7 ± 0.4	19.3 ± 4.2
18:0	1.2 ± 0.3	1.3 ± 0.5	1.0 ± 0.0	1.8 ± 0.7
18:1	3.8 ± 0.3	3.5 ± 0.8	3.6 ± 0.5	4.9 ± 0.6[b]
18:2	2.1 ± 0.5	2.3 ± 0.9	2.1 ± 0.8	2.9 ± 0.6
Other[c]	0.2 ± 0.3	0.1 ± 0.2	2.3 ± 0.6	0.9 ± 0.5

[a]Alveolar subfractions (P10, P60, P100) were prepared as described in Table 2. Lamellar bodies were prepared from the post-lavage lung tissue. The results are expressed as percentage by weight (± 1 SD) of the total fatty acid methyl esters. Fatty acid methyl esters were identified by reference to known methyl ester standards.
[b]Significantly different from alveolar subfractions ($P < 0.05$ by Duncan's Multiple Range Test).
[c]Contains 20:4 and other unidentified long chain constituents.

TABLE 5

Phosphatidylglycerol Fatty Acyl Composition of Mouse Surfactant Subfractions[a]

Fatty acid	P10 (2)	P60 (2)	P100 (3)	Lamellar bodies (5)
14:0	0.5 ± 0.6	4.2 ± 2.0	1.2 ± 1.0	2.3 ± 1.5
16:0	57.3 ± 6.2	56.9 ± 9.6	58.1 ± 4.7	53.8 ± 3.4
16:1	8.5 ± 1.6	6.0 ± 2.8	5.1 ± 3.4	6.8 ± 2.6
18:0	5.1 ± 1.2	7.5 ± 3.5	4.8 ± 1.4	4.6 ± 1.5
18:1	19.8 ± 2.4[b,c]	12.7 ± 2.1	17.6 ± 1.3[c]	15.8 ± 1.4
18:2	8.2 ± 2.5	6.3 ± 3.0	7.9 ± 0.6	8.8 ± 1.3
Other[d]	0.8 ± 1.1	6.5 ± 1.8	5.4 ± 0.5	8.0 ± 4.1

[a]Alveolar subfractions and lamellar bodies were prepared as indicated in Table 3. Expression of results and statistical analysis are also as described for Table 3.
[b]Significantly different from lamellar bodies.
[c]Significantly different from P60.
[d]Includes 20:4 and other unidentified long chain constituents.

TABLE 6

Phospholipid Distribution over Alveolar Surfactant Subfractions: Mouse *vs.* Rabbit[a]

Fraction	% Total alveolar lavage phospholipid[b]	
	Mouse	Rabbit
Pc	10.9 ± 3.5	9.7 ± 4.5
P10	26.6 ± 3.2	48.4 ± 9.9[c]
P60	24.0 ± 4.6	12.9 ± 6.1[c]
P100	27.1 ± 2.4	20.9 ± 10.1
S100	11.3 ± 2.0	8.1 ± 4.2

[a]Alveolar surfactant subfractions, pellets, P10, P60, P100, and the resultant supernatant, S100, were prepared as described in the legend to Figure 1; following a preliminary centrifugation for 5 min at 140 × *g* to harvest a cellular pellet (Pc).
[b]Each value represents the mean ± 1 SD for six determinations in mice and seven in rabbits.
[c]Significantly different from mouse ($P < 0.01$ by Student's *t*-test).

esterified to PG in both the alveolar subfractions and the isolated lamellar bodies was 16:0 (Table 5), although its relative proportion of the total fatty acids was considerably less for PG than for PC. Although 18:1 constituted a minor PC fatty acid, it appeared to represent the second most abundant PG fatty acid. Of the samples analyzed, P60 appeared to contain the least amount of this fatty acid.

The total phospholipid content of unprocessed mouse alveolar lavage fluid was 1083.4 ± 69.9 μg/g lung, of which 88.2 ± 4.0% could be accounted for following fractionation. For rabbits, these values were 1263.6 ± 260.6 μg phospholipid/g lung and 87.3 ± 4.5% recovery. The quantity of phospholipid recovered in the individual subfractions was consistent in both species. However, significant differences were found between the two species in the pattern of distribution of phospholipid over the individual subfractions (Table 6). The cellular pellet comprised approximately 10% of the total recoverable phospholipid in both species and the lavage 100,000 × *g* supernatant (S100) another 10%, but in the mouse the sedimentable surfactant phospholipid was more or less evenly distributed over the remaining individual subfractions, whereas in the rabbit almost 50% of the total recoverable phospholipid was present in the most easily sedimentable subfraction, P10, with less than 15% in the intermediate fraction, P60.

DISCUSSION

Previous studies in rats (7) and rabbits (4,6) have shown that pulmonary surfactant obtained from alveolar lavage can be separated by differential centrifugation into subtypes or subfractions that have distinctive morphologies as well as different compositions, surface properties and labelling patterns. Though a variety of centrifugation schemes have been employed to isolate these subfractions, it can nevertheless be generalized that the most easily sedimented fractions, obtained as pellets by using g forces up to 10,00 × *g*, consist of large multilamellated structures which are typical of newly or recently secreted lamellar bodies, whereas the most difficult to sediment fractions, requiring g forces up to 100,000 × *g* for as long as 15 h, consist of much smaller unilamellar vesicles which are believed to represent catabolic end products of the secreted surfactant; and that pelleted material obtained under centrifugal conditions intermediate between these extremes contains intermediate metabolic forms of the alveolar surfactant (for a review, see Ref. 10).

In our laboratory we have recently adopted a three-step differential centrifugation scheme for the study of alveolar surfactant subfractions in fetal, newborn and adult rabbits. After an initial 5-min centrifugation to remove a cellular pellet, this scheme involves: (i) a 30-min centrifugation at 10,000 × *g*, which we had previously found to pellet virtually all of the large multilamellated structures typical of newly secreted surfactant present in fetal and newborn lung (23); (ii) a 60-min centrifugation at 60,000 × *g*, which has been shown by other investigators to harvest intermediate alveolar surfactant forms (4,6,7); and (iii) a 15-h centrifugation at 100,000 × *g* to harvest the small unilamellar vesicles which are present in newborn and adult (4,6), but not fetal (5,6,23), lung. By applying this fractionation scheme to lavage fluid obtained from

adult mice, we were able to identify at least two morphologically distinct subfractions, namely, the large multilamellar vesicles present in the 10,000 × *g* pellet (P10) and the small unilamellar vesicles present in the 100,000 × *g* pellet (P100). Both of these fractions were characteristically similar to those described by ourselves (23) and others for rabbits (4,6) and rats (7) using appropriately comparable conditions of centrifugation. The fraction which we designate as P60 is less well defined than P10 and P100. It appears to contain a mixture not only of the structures found in P10 and P100, but also of structures which are not present in either of these two subfractions. Another distinguishing feature of P60 is its phospholipid/protein ratio, which is significantly greater than that of any of the other subfractions. These observations suggest that P60 may indeed be distinct from the other two pellet fractions and it may, in fact, represent a metabolic form that is intermediate between them. This would be in agreement with previously reported findings in other species (4,6,7).

An interesting observation along this line is a recent finding (to be reported in a separate communication) in our laboratory that smoke exposure, which had previously been shown to increase the total alveolar surfactant phospholipid pool nearly twofold (21), had no effect on the phospholipid content of this P60 pellet but significantly increased the other lavage subfractions. This suggests the possibility of an important regulatory role for this intermediate fraction in the alveolar processing of secreted surfactant. This concept is supported by the recent studies of Gross (15,16), which suggest that the generation of this subfraction may be catalyzed by an enzyme, which this referred to as convertase.

The latter studies have also isolated and identified three alveolar surfactant subtypes in mouse lavage fluid. It was shown that these subtypes were in metabolic sequence and that metabolic conversions from one form to another could be induced *in vitro* (9). The subtype referred to as "light" would correspond to our P100 subfraction, whereas the fractions designated as "ultra heavy" and "heavy" would correspond to our P10 and P60 subfractions. One difference between our studies and those of Gross and Narine (8,9) is that they found tubular myelin figures in their "ultra heavy" and "heavy" subfractions, whereas we did not find these figures in any of our subfractions. The absence of tubular myelin could be due to structural rearrangement during the isolation procedure (34) or the absence of calcium ions, which might be required to preserve these structures (35), from the lavage and isolation procedures. Another possibility is that these structures were present in the low-speed pellet (Pc) which we did not examine by electron microscopy. The major difference between our study and that of Gross and Narine (8,9) is that they employed a technique involving centrifugation to equilibrium on continuous sucrose gradients, a somewhat more tedious and time-consuming (requiring 60 h to reach equilibrium) procedure than the differential centrifugation scheme used for the present study. It also makes interspecies comparisons difficult, given that most reported studies have employed a stepwise differential centrifugation scheme to prepare individual subtypes or subfractions (4,6,7).

In the present study in which we applied the same isolation technique to lavage samples obtained from both adult mice and rabbits, we found highly reproducible and consistent phospholipid recoveries in the individual surfactant subfractions obtained from both species. The difference in distribution of phospholipid over the subfractions in the two species suggests that there may be species differences in the rates of subtype conversion and/or uptake. The low levels of P10 present in mice, which is in agreement with previous findings of Gross and Narine (8) in mice and Spain *et al.* (7) in rats, in comparison to the much higher levels present in the rabbit, as reported in this and other (4,6,18) studies suggests that the first step in the metabolic processing of the newly secreted surfactant may occur more readily in murine than in rabbit lung. Whether or not this could be due to inherent properties of the surfactants is not known. There are only slight interspecies differences in the overall phospholipid composition and fatty acyl composition of some of the individual phospholipids, as shown in this and other (4,8) reports. However, the interspecies differences observed in SP-A (36,37), the major surfactant-specific protein, which has been suggested to play a role in the unwinding of newly-released surfactant, could possibly contribute to this effect. Another surfactant-associated protein which may contribute to this effect is the newly-identified SP-D (38), which has recently been shown to counteract the effects of SP-A (39) and appears to be differentially distributed among the lavage subfractions in at least two species (rat and bovine) studied to date (40). Studies are in progress in our laboratory to investigate these possibilities.

The differences that we and others (4,8) observed in the surface activity among the three alveolar surfactant subtypes might also be attributable to differences in composition. While there are only slight differences in the phospholipid composition of these fractions in both mice and rabbits (4,8) there appear to be differences in protein composition. For example, it has been shown (4–6) that the least surface-active fraction obtained from rabbits, which is comparable to our P100 subfraction, is virtually devoid of SP-A, which, in addition to its function discussed above, has been purported to play at least an ancillary role in the expression of surface activity (for a review, see Ref. 37). Preliminary studies in our laboratory suggest the absence of SP-A not only from the P100 subfraction but also from P60. The surface activity of the latter fraction might thus be attributable to its high proportion of phospholipid to protein as observed in the present study. Further studies are required to clarify these issues.

In summary, we have identified three subfractions or subtypes of mouse alveolar surfactant that can be quantitatively and reproducibly harvested by a three-step differential centrifugation scheme. These preliminary studies provide us with an excellent model for extensively studying surfactant metabolism in this species. We have also demonstrated differences in the distribution of phospholipids over these subfractions obtained from the mouse and rabbit, suggesting the possibility of interspecies differences in the rates of conversion and/or uptake of secreted surfactant.

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The Role of Arginines in Stabilizing the Active Open-Lid Conformation of *Rhizomucor miehei* Lipase

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Molecular dynamics simulations for the lid covering the active site of *Rhizomucor miehei* lipase [EC 3.1.1.3] postulated that, among other interactions, Arg86 in the lid stabilized the open-lid conformation of the protein by multiple hydrogen bonding to the protein surface. Chemical modification of arginine residues in *R. miehei* lipase with 1,2-cyclohexanedione or phenylglyoxal resulted in residual activities in the hydrolysis of tributyrin of 66 and 46%, respectively. Tryptic maps of native and phenylglyoxal-reacted *R. miehei* lipase showed that Arg86 was the residue modified most, when the lipase was inhibited to the greatest extent. Guanidine, a structural analog to an arginine side chain, inhibited both the native enzyme and the arginine-modified enzymes, resulting in residual activities of 26% as compared to the native enzyme. The inhibition was not an effect of enzyme denaturation. The native enzyme was also inhibited by 1-ethylguanidine, benzamidine and urea, but to a lesser degree than by guanidine. Lipases from *Humicola lanuginosa* and porcine pancreas in 100 mM guanidine showed residual activities of 88 and 70%, respectively. The lipases from *Candida antarctica, C. rugosa, Pseudomonas cepacia* and *P. fluorescens* were not inhibited by guanidine. The inhibition of *R. miehei* lipase by structural analogs of the arginine side chain and after chemical modification of arginine residues suggest a role of an arginine residue in stabilizing the active open-lid conformation of the enzyme.
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Lipases [EC 3.1.1.3] need the hydrophobic interface formed by their water insoluble ester substrates to express full catalytic activity in hydrolytic systems (1–3). The interfacial activation has been proposed to be due to a conformational change in the enzyme (2,4). The three-dimensional structures of the lipases from *Rhizomucor miehei* (5), human pancreas (6) and *Geotrichum candidum* (7) have been published. These enzymes all have an active site hidden under a lid which must be displaced to expose the active site of the enzyme to the substrate. The three-dimensional structures of two *R. miehei* lipase-inhibitor complexes have been solved, showing the displacement of the lid (8,9). In these complexes the lid exposes its hydrophobic side to the substrate, and its hydrophilic side is buried in a polar cavity of the enzyme (8,9). Asn87 in the lid has been shown to form new interactions with the polar cavity when the lid is open (9). Other polar residues in the lid might also stabilize the active open-lid conformer of the enzyme.

In this paper we report the effects of chemical, arginine-specific modifications of *R. miehei* lipase and the results of inhibition studies with structural analogs to the side chain of arginine.

MATERIALS AND METHODS

Chemicals. Tributyrin, dithiothreitol, bovine serum albumin, 2-{[2-hydroxy-1,1-*bis*(hydroxymethyl)ethyl]amino}-ethanesulfonic acid (TES) and gum arabic were purchased from Sigma (St. Louis, MO). 1,2-Cyclohexanedione, phenylglyoxal, guanidine hydrochloride, 1-ethylguanidine hydrochloride, benzamidine hydrochloride hydrate, iodoacetamide, trichloroacetic acid and trifluoroacetic acid (spectrophotometric grade) were from Aldrich-Chemie (Steinheim, Germany). [7-^{14}C]Phenylglyoxal with a specific activity of 855 KBq/mol was obtained from Amersham (Buckinghamshire, England). Urea, ammonium bicarbonate and calcium chloride were obtained from Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

Enzymes. Highly purified lipase [EC 3.1.1.3] from *R. miehei* (7800 units/mg), *Humicola lanuginosa* (5400 units/mg), *Pseudomonas cepacia* (3400 units/mg), *Candida antarctica* A (180 units/mg) and *C. antarctica* B (340 units/mg) were generous gifts from Novo Nordisk A/S (Bagsvaerd, Copenhagen, Denmark). No contaminants could be detected in the *R. miehei* lipase after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis. One unit of lipase produces 1 μmol butyric acid per minute from tributyrin at pH 7.0 at 25°C.

Commercial lipase from *C. cylindracea* (recently reclassified as *C. rugosa*) [1010 units/mg solid (7770 units/mg protein)] and porcine pancreas [53 units/mg solid (143 units/mg protein)] were obtained from Sigma. These enzymes were purified from insoluble material by collecting the supernatant after centrifugation of crude enzyme dissolved in 10 mM TES, pH 7.5. One unit of lipase splits off 1 μmol of fatty acid from olive oil in one hour at 37°C and pH 7.2 *(C. rugosa)* or pH 7.7 (porcine pancreas).

Lipase from *P. fluorescens* (31.5 units/mg) was purchased from Fluka (Buchs, Switzerland). One unit of lipase liberates 1 μmol oleic acid per minute from triolein at pH 8.0 and 40°C.

Trypsin [EC 3.4.21.4] (sequencing grade) was obtained from Boehringer Mannheim GmbH (Mannheim, Germany).

Enzyme activity assay. The substrate solution containing tributyrin (0.10 M), calcium chloride (0.10 M) and gum arabic (5%, wt/vol) in water, was emulsified by sonication for one minute. The pH was adjusted to 7.0 with sodium hydroxide (100 mM). The solution (2.0 mL) was equilibrated for 2 min in a stirred, thermostatted pH-stat cuvette at 25°C. The enzyme (*R. miehei* 11–17 μg/mL, *H. lanuginosa* 54 μg/mL, porcine pancreas 50 mg/mL,

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Abbreviations: CD, circular dichroism; DHB, 2,5-dihydroxy-benzoic acid; HPLC, high-performance liquid chromatography; LDMS, laser desorption mass spectrometry; PDMS, plasma desorption mass spectrometry; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-*bis*(hydroxymethyl)ethyl]-amino}ethanesulfonic acid; UV, ultraviolet.

C. antarctica A 0.40 mg/mL, *C. antarctica* B 0.22 mg/mL, *C. rugosa* 6.4 mg/mL, *P. cepacia* 35 μg/mL and *P. fluorescens* 2.3 mg/mL) was dissolved in 10 mM TES buffer, pH 7.5. The reaction was started by the addition of 50 μL enzyme solution to the stirred, thermostatted cuvette. The activity was determined under nitrogen using a Radiometer (Copenhagen, Denmark) pH-stat, equipped with an ABU91 autoburette (1 mL) connected to a VIT90 videotitrator. The pH was automatically maintained at 7.0 with sodium hydroxide (100 mM). The reactions were run for five minutes. In experiments with inhibitors, the inhibitor was added to both the substrate emulsion and the enzyme solution before the assay was performed. The coefficients of variation for the activity determinations were 3–8% (3–7 determinations on 8 samples) with the same substrate preparation and 1–18% (4–5 determinations on 3 samples) with different substrate preparations.

Modification of arginine residues. The *R. miehei* lipase [100 μM, $\varepsilon = 47000\ M^{-1}\ cm^{-1}$ at 280 nm (Novo-Nordisk A/S)] was incubated with 1,2-cyclohexanedione (30 mM) in 0.2 M sodium borate, pH 9.0, at 37°C (10) or the enzyme (4 μM) was treated with phenylglyoxal (3 mM) in 0.2 M sodium bicarbonate, pH 8.2, at 25°C (11). All reactions were performed in the dark. Under the conditions described, both 1,2-cyclohexanedione (10) and phenylglyoxal (11,12) are highly specific for arginine residues. Phenylglyoxal also reacts with free α-amino groups to give a residue of the corresponding α-keto acid as a side reaction (11). Reference lipase samples were treated and incubated as described above, omitting the modifying reagent. The reaction mixtures (1.0–1.7 mL) were incubated in an end-over-end incubator. Samples (50 μL) were removed for assay of enzymatic activity. The modified enzyme was purified from excess reagent by gel filtration through a PD-10 column (Sephadex® G-25 M, bed vol 9.1 mL, Pharmacia Biotechnology, Uppsala, Sweden) equilibrated with 10 mM TES, pH 7.5.

Stoichiometry of [7-^{14}C]phenylglyoxal to lipase. *R. miehei* lipase (4 μM) was incubated with [7-^{14}C]-phenylglyoxal (3 mM; specific activity 11 KBq/mol) under the same reaction conditions as described above, in a reaction volume of 100 μL. After the specified reaction time, bovine serum albumin (100 μL, 1 mg/mL, previously treated with a 500-fold molar excess of unlabeled phenylglyoxal) was added as a nonspecific protein carrier. The incorporation of [7-^{14}C]phenylglyoxal into *R. miehei* lipase was determined by precipitation of the protein by the addition of trichloroacetic acid (60 μL, 50% wt/vol). The precipitates were collected by centrifuging the mixture at 8000 × *g* for 30 min and washed (3 × 0.5 mL) with trichloroacetic acid (5% wt/vol). The pellets were resuspended in 8 M urea and transferred to scintillation vials, and the radioactivity was measured in 10 mL of Ready safe™ (Beckman Instruments Inc., Fullerton, CA). The instrument used was a Packard Tri-Carb® 1500 liquid scintillation analyzer (Downers Grove, IL).

Proteolytic digestion. Native (150 μg) or arginine-modified *R. miehei* lipase (150 μg) was dissolved in 50 μL 8 M urea and 0.4 M ammonium bicarbonate. Dithiothreitol (5 μL, 45 mM) was added, and the mixture was kept at 50°C for 15 min. After cooling to room temperature, iodoacetamide (5 μL, 100 mM) was added, and the solution was incubated at room temperature for 15 min. After dilution with water (140 μL), trypsin (10 μg in 10 μL 0.01% trifluoroacetic acid) was added. The reaction mixture was incubated at 37°C for 24 h. The peptides produced were separated on a reversed-phase high-performance liquid chromatography (HPLC) column.

Chromatography. The proteolytic digests were analyzed by reversed-phase HPLC on a C18-column (Vydac 218 TP, 4.6 × 250 mm, 5 μm, Hesperia, CA). The chromatograms were developed at 25°C with a gradient of an increasing concentration of acetonitrile. Mobile phase A consisted of trifluoroacetic acid/water (0.1:100, vol/vol) and mobile phase B was acetonitrile/mobile phase A (60:40, vol/vol). The gradient was designed as follows: 0–5 min, 0% B (100% A); 5–45 min, 0–45% B; 45–100 min, 45–55% B; 100–145 min, 55–100% B; 145–150 min, 100% B; 150–155 min, 0% B. The flow rate was 0.5 mL/min. Fractions were collected and peptides were identified by plasma desorption mass spectrometry (PDMS). The HPLC system consisted of two pumps (LKB, model 2150), a gradient programmer (LKB, model 2152), a Rheodyne injection valve with a 100 μL sample loop. Peptides were detected with an ultraviolet (UV) detector (214 nm; LKB, model 2140) or an FS 970 L.C fluorimeter (excitation wavelength 280 nm, emission filter 270 nm; Schoeffel Instrument GmbH, Trappenkamp, Germany). Data were collected with a Macintosh SE personal computer equipped with the software Dynamax® (Rainin Instrument Company Inc., Woburn, MA).

Mass spectrometry. PDMS analyses were performed on lyophilized HPLC fractions containing the relevant peptides, using a Bio-Ion 20 instrument (Bio-Ion AB, Uppsala, Sweden) and the nitrocellulose foil-spin drying technique as published by Jardine (13).

Laser desorption mass spectrometry (LDMS) was carried out directly on the lyophilized mixture of tryptic peptides. The instrument used was a LDI-1700 Mass Monitor (Linear Scientific Inc., Reno, NV). In order to achieve good mass dynamic ranges, experiments were performed using 2,5-dihydroxy-benzoic (DHB) acid and sinapinic acid as matrices. DHB was best suited for low molecular weight constituents (<2 kDa) while sinapinic acid worked well for high-molecular weight peptides. DHB solutions were 100 mM in 10% (vol/vol) aqueous ethanol. Sinapinic acid solutions were 100 mM in 40% (vol/vol) acetonitrile in 10% (vol/vol) aqueous ethanol. The lyophilized peptide mixtures were reconstituted using 250 μL of 0.1% (vol/vol) trifluoroacetic acid in 50% (vol/vol) aqueous acetonitrile. Samples were mixed with matrix at 1:3 or 1:4 dilutions. For the most part, molecular weights were determined using an external standard based upon a calibration mixture of vasopressin, calcitonin, synacthen and hirudin. In some experiments, the samples were spiked with this solution to function as an internal standard.

Determination of enantioselectivity. Hydrolysis of racemic heptyl 2-methyldecanoate was done in a pH-stat. After 30% conversion, the enantiomeric excess (14) of the 2-methyldecanoic acid produced was determined. The enantiomeric ratio (E) was calculated according to Chen *et al.* (15). The synthesis of the substrate (16) work-up procedures (16) and the determination of the enantiomeric excess (17) have been described elsewhere.

Circular dichroism. CD spectra (205–250 nm) of *R. miehei* lipase (0.10 mg/mL in 10 mM TES, pH 7.0) were recorded at 25°C in the presence of guanidine (0–4.0 M),

using a JASCO J-720 (Tokyo, Japan) spectropolarimeter. The fraction of folded protein was determined at 222 nm.

RESULTS

Chemical modification of arginines. Arginine-specific chemical modification of *R. miehei* lipase with 1,2-cyclohexanedione or phenylglyoxal, resulted in residual activities of 66 and 46%, respectively, as compared to untreated enzyme, with tributyrin as substrate (Fig. 1). The reduction of activity during arginine modification plateaued in three hours with both reagents.

The amount of ^{14}C-labeled phenylglyoxal bound to *R. miehei* lipase was determined by trichloroacetic acid precipitation. These labeling studies showed that approximately 8 mol of phenylglyoxal was bound to 1 mol of the enzyme, when the lipase had been inhibited to the full extent achieved here.

Identification of modified arginines. Tryptic digestion of native *R. miehei* lipase and subsequent LDMS analyses of the peptide mixture showed that all the peptides predicted were produced. The masses recorded were all within 0.6% of the expected values (data not shown).

Phenylglyoxal-reacted arginine residues in proteins are known to be resistant to tryptic cleavage (11). Tryptic peptides of native lipase were separated on an HPLC system and identified by PDMS analyses (Table 1 and Fig. 2). The comparison of peak heights of specific tryptic peptides, produced from native and phenylglyoxal treated lipase, showed that a number of arginines in the lipase had reacted with phenylglyoxal (Fig. 2). Arg86 (based on the absence of T9) was the residue modified to the highest degree (>85%). Arg30 (T3), Arg68 (T5) and Arg160 (T13) were all found to be modified to a significantly lower extent (<70%) than Arg86 in the inhibited lipase. The degrees of modification based on fluorescense detection were 58% for Arg30 (T3), 56% for Arg68 (T5) and 85% for Arg86 (T9) (data not shown). The quantification in the fluorescense chromatogram was more reliable for these peaks because of lower noise and fewer peaks than in the UV chromatogram. For several other arginines, it could be shown that they were only partially modified when the lipase was inhibited to the full extent achieved. It was shown by the combined disappearance of T17 (70%) and T18 (30%) that both Arg197 and Arg202 had reacted to some extent (40 and 30%, respectively). According to the constant peak height of peptide T1, Arg7 was not modified. Arg178 was not modified since peptides T13 and T14 were similarly decreased, due to modification of Arg160 (T13). The degree of modification of Arg80 (T7) and Arg196 (T15) could not be unambiguously determined as T2, T7 and T15 were coeluted. But based on the total amount of incorporated phenylglyoxal, it can be concluded that none of the residues Arg80 or Arg196, alone or together, were modified to more than 60 mol%.

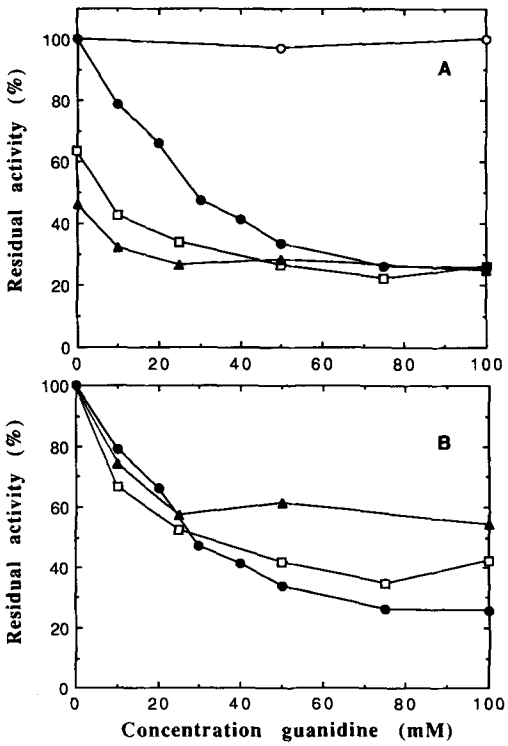

FIG. 1. Residual activity of native (●,○), 1,2-cyclohexanedione-reacted (□) and phenylglyoxal-reacted (▲) *Rhizomucor miehei* lipase as a function of guanidine concentration. Guanidine was present in both the enzyme solution and the substrate emulsion (●, □, ▲) or only in the substrate emulsion (○), before mixing enzyme and substrate. Activity was determined with tributyrin as substrate at pH 7.0 and 25°C. The experimental points are mean values of repeated experiments. In A, 100% residual activity corresponds to the activity of native enzyme in the absence of guanidine. In B, 100% residual activity corresponds to the activity in the absence of guanidine of native lipase or the activity of lipase obtained after chemical modification.

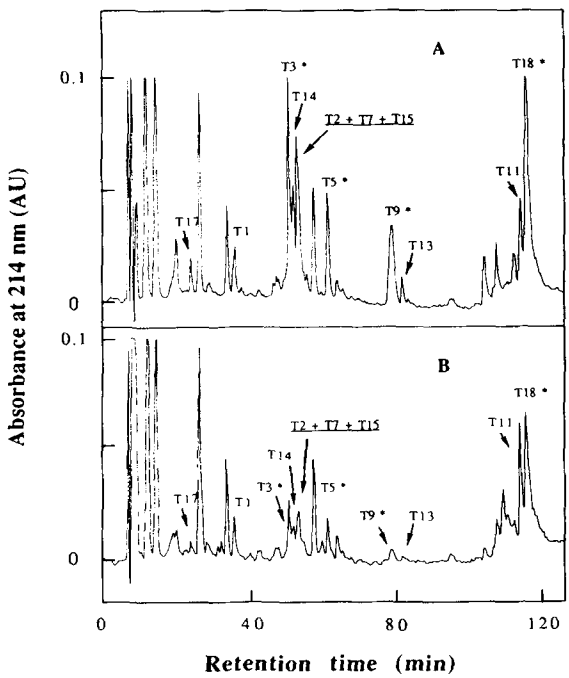

FIG. 2. Reversed-phase high-performance liquid chromatography chromatograms of tryptic peptides from control (A) and phenylglyoxal-reacted (B) *Rhizomucor miehei* lipase. Eight mol phenylgloxal (B) were incorporated into 1 mol lipase. Peptides were identified by plasma desorption mass spectrometry analyses. The chromatograms were recorded with a ultraviolet detector (λ 214 nm). The peptides detected with a fluorescence detector are indicated with an asterisk (*). Decreased peaks (B compared to A) not outlined contained peptides not recognized as tryptic peptides from *R. miehei* lipase.

TABLE 1

Tryptic Peptides of *Rhizomucor miehei* Lipase[a]

Peptide	Residues	Mass MH^+ (Da)		Sequence
		Predicted[b]	Experimental[c]	
T1	1-7	717.4	717.3	SIDGGIR
T2	8-30	2656.2[d]	2656.8	AATSQEINELTYYTTLSANSYCR
T3	31-50	2302.1[d]	2298.0	TVIPGATWDCIHCDATEDLK
T4	51-53	373.3		IIK
T5	54-68	1741.9	1741.2[e]	TWSTLIYDTNAMVAR
T6	69-73	535.2		GDSEK
T7	74-80	911.5	911.5	TIYIVFR
T8	81-86	606.3		GSSSIR
T9	87-106	2191.1	2191.1	NWIADLTFVPVSYPPVSGTK
T10	107-109	383.2		VHK
T11	110-131	2472.2	2472.2	GFLDSYGEVQNELVATVLDQFK
T12	132-137	785.4		QYPSYK
T13[f]	138-160	2386.3[d]	2385.8	VAVTGHSLGGATALLCALDLYQR
T14	161-178	2026.0	2025.3[g]	EEGLSSSNLFLYTQGQPR
T15	179-196	1926.0		VGDPAFANYVVSTGIPYR
T16	197-197	175.1		R
T17	198-202	618.3	618.6	TVNER
T18	203-269	7441.6[d]		DIVPHLPPAAFGFLHAGEEYWITDN SPETVQVCTSDLETSDCSNSIVPFT SVLDHLSYFGINTGLCT

[a]Theoretical tryptic peptide sequences (T1-T18) and masses were based on the published lipase sequence (18; see also footnote f). The experimentally determined peptide masses were used to assign the peaks in the high-performance liquid chromatography (HPLC) chromatogram (Fig. 2).
[b]Peptide mass was calculated using the computer software MacProMass 1.0; Vemuri, S., and Lee, T.D., Division of Immunology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, CA 91010.
[c]Peptide mass was experimentally determined by plasma desorption mass spectrometry analyses of HPLC-separated peptides.
[d]Mass was corrected for the alkylation of cystein-residues.
[e]This peptide was also detected as the methionine oxidized species (MH^+ 1756.9 Da).
[f]Residue 156 has incorrectly been assigned as G (Gly) in the published lipase sequence (Ref. 18) and should be D (Asp) according to the Protein Data Bank at Brookhaven (Ref. 19), entry number 1TGL.
[g]This peptide was also detected as an alkylated species (MH^+ 2081.5 Da).

Inhibition by arginine side chain analogs. Native enzyme and 1,2-cyclohexanedione- or phenylglyoxal-treated enzymes were all inhibited by guanidine. The enzyme and the substrate solutions had to be mixed separately with guanidine before the reaction was started by the addition of enzyme to the substrate (Fig. 1). The same residual activity, 26%, was obtained either by guanidine inhibition of native enzyme or chemical arginine-modification plus guanidine inhibition of native enzyme (Fig. 1A). The concentrations of guanidine which resulted in half of maximum inhibition, the apparent inhibition constants were 20 mM and 10 mM for the native and arginine-modified enzymes, respectively (Fig. 1B). The inhibition produced by guanidine was not an effect of enzyme denaturation, as ascertained by circular dichroism spectroscopy at 222 nm, pH 7.0, at 25°C. The enzyme was found to have an intact secondary structure in 0.5 M guanidine. The enzyme was partially and fully unfolded in 1.0 M and 4.0 M guanidine, respectively.

The native enzyme was also inhibited by 1-ethylguanidine, benzamidine or urea, but their inhibiting effects were lower than that of guanidine (Table 2).

Guanidine reduced the enantioselectivity of the enzyme. With racemic heptyl 2-methyldecanoate as substrate, the enantiomeric ratio [$E = (V_{max}/K_m)_S/(V_{max}/K_m)_R$, where S and R denote the enantiomers (15)] changed from 8.5 (no inhibitor) to 1.9 in the presence of 100 mM guanidine.

Lipases from porcine pancreas and *H. lanuginosa* were also inhibited by guanidine under the conditions described above, showing residual activities of 70 and 88%, respectively, in 100 mM guanidine (Table 3). In contrast, lipases from *C. antarctica* (A and B), *C. rugosa*, *P. cepacia* and *P. fluorescens* were not inhibited by guanidine (Table 3).

DISCUSSION

It has recently been shown that Asn87 in the lid of *R. miehei* lipase interacts with a polar cavity of the enzyme

TABLE 2

Residual Activity of *Rhizomucor miehei* Lipase in the Presence of Inhibitors

Inhibitor	Residual activity[a] (%)
Guanidine	26 ± 5
1-Ethylguanidine	58 ± 8
Benzamidine	56 ± 11
Urea	78 ± 7

[a]Reference samples were assayed in the absence of inhibitor. Activity was determined with an inhibitor concentration of 100 mM and tributyrin as substrate at pH 7.0 at 25°C. SD is based on three separate experiments.

TABLE 3

Residual Activity of Different Lipases in the Presence of 100 mM Guanidine

Lipase	Residual activity[a] (%)
Rhizomucor miehei	26 ± 5[b]
Porcine pancreas	70 ± 9[b]
Humicola lanuginosa	88 ± 2[b]
Candida antarctica A	97
C. antarctica B	102
C. rugosa	101 ± 3[c]
Pseudomonas cepacia	95
P. fluorescens	95

[a]Reference samples were assayed in the absence of guanidine. Activity was determined with tributyrin as substrate at pH 7.0 at 25°C.
[b,c]SD is based on three and two separate experiments, respectively.

when the lid is open (9). Other polar residues in the lid might also stabilize the active open-lid conformer of the enzyme. Three arginine residues in the structure, Arg30, Arg80 and Arg86, have been reported to have multiple conformations in the open lipase-inhibitor complex (9). Arg86 is located in the lid whereas the side chains of Arg30 and Arg80 are situated in the region referred to as the polar cavity.

Molecular dynamics simulations on *R. miehei* lipase, starting from the closed-lid conformer (Fig. 3A) (5), indicated that Arg86 in the lid could be an important residue in the stabilization of the open-lid conformation (20). Running the simulation on the enzyme where Arg86 had been mutated to an alanine residue showed a significantly decreased stabilization of the open-lid conformation (20).

Tryptic maps of native and phenylglyoxal-reacted *R. miehei* lipase showed that Arg86 was the amino acid that had been modified to the highest degree while Arg30, Arg68 and Arg160 were modified to a lesser degree in the inhibited lipase. Arg7, Arg80, Arg178, Arg196, Arg197 and Arg202 were not, or only to a small extent, modified. This is consistent with the result of the ^{14}C-phenylglyoxal labeling experiments which indicated that either four arginines were modified quantitatively or that more than four arginines were modified partially, since two phenylglyoxal molecules react with one arginine (21). The finding that Arg86 was modified most when the lipase was inhibited to the fullest extent and that further incubation with phenylglyoxal did not inhibit the lipase further, suggests that the modification of Arg86 plays an important role in the inhibition of the lipase. Arg30 and Arg80 are located in a region of the lipase structure where the polar side of the lid interacts with the protein surface in the open form (Fig. 3). It seems reasonable to assume that the modification of these arginines also could affect the stabilization of the open-lid conformation. Arg68 and Arg160 are located far away from the lid and the active site of the lipase, suggesting that the modification of these residues should not affect enzyme activity. To unambiguously determine which arginines are important for enzyme activity, specific mutants of the enzyme are needed. Unfortunately no such mutants have been obtained to date.

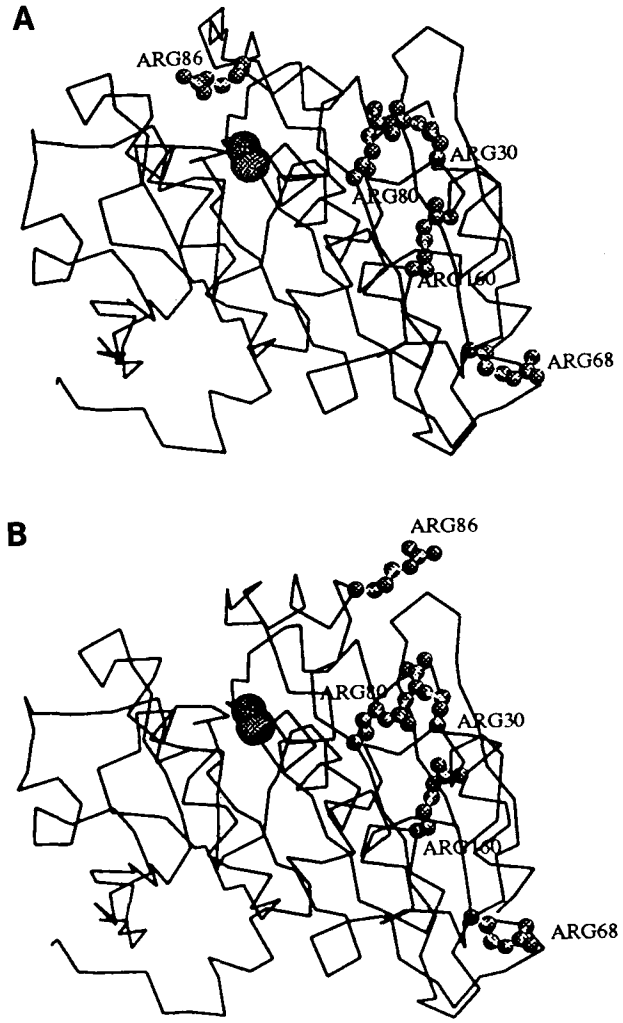

FIG. 3. Structures showing the positions of Arg30, Arg68, Arg80, Arg86 and Arg160 in the closed form (A) (5) and the open form (B) (9) of the *Rhizomucor miehei* lipase. The van der Waals surface of the active site residue Ser144 is shown dotted.

Modification of Arg86 with 1,2-cyclohexanedione or phenylglyoxal should not prevent Arg86 from forming hydrogen bonds or ionic bonds, but the arginine residue would become more bulky, which could affect lid opening and enzyme activity. The lower residual activity obtained after phenylglyoxal treatment, may be an effect of such a bulkier product (11) formed with this reagent, compared to that obtained by 1,2-cyclohexanedione treatment (10).

Guanidine, structurally resembling the side chain of an arginine residue, might specifically compete with Arg86 for its binding site and thus destabilize the active open-lid conformation of the enzyme. The order in which substrate, enzyme and guanidine were mixed was essential. When guanidine was present only in the substrate solution, which affects the final concentration very little, no inhibition was seen. But when guanidine was added to both the enzyme solution and the substrate, the lipase was inhibited to the full extent achieved here (Fig. 1A). Probably the enzyme obtained its active conformation at the substrate interface faster than guanidine was bound to

TABLE 4

Amino Acid Sequences of the Lid Region of Different Lipases

Lipase	Amino acid sequence	Residues	Reference
Rhizomucor miehei	GSSSIRNWIADL	81-92	(8)
Porcine pancreas	CQKNILSQIVDIDGIWEGTRDFVAC	237-261	(6)
Humicola lanuginosa	GSRSIENWIGNL	82-93[a]	(22)
Candida rugosa	PEGTYEENLP	65-74	
	and/or		
	SDTLEDATNNTPGFLAY	283-299	(7)

[a]The possible lid region of *H. lanuginosa* lipase was identified after alignment of the primary sequence to the *R. miehei* lipase sequence. These enzymes show about 32% sequence identity.

the enzyme. Once the enzyme attained its open-lid conformation at the substrate interface, guanidine could not remove Arg86 from its binding site. However, when the enzyme solution but not the substrate emulsion contained guanidine, the extent of inhibition varied from a full effect to a moderate one, in spite of a substantial dilution of the inhibitor. The degree of inhibition would be correlated to the fraction of enzyme molecules having guanidine bound, at the moment of adsorption, to the substrate interface.

The same residual activity was obtained by guanidine inhibition of native enzyme or chemical arginine-modification plus guanidine inhibition, showing that inhibition produced by guanidine and the chemical modification of arginine residues were complementary. The lower apparent inhibition constants of guanidine for the arginine-modified enzymes compared to the native enzyme is consistent with the hypothesis that both arginine-modification and the presence of guanidine hinder the interaction of Arg86 with its binding site.

The hydrophobic interaction between substrate and the hydrophobic side of the lid, exposed in the open-lid conformation, stabilizes the enzyme in an open form, as described by Brzozowski *et al.* (8) and Derewenda *et al.* (9). Arg86 may take part in anchoring the lid in its optimal position for catalysis. When Arg86 was prevented from binding its hydrogen bonding site, due to the presence of guanidine or after chemical modification, the lid was not in its optimal position, resulting in lower activity and altered enantioselectivity. The altered enantioselectivity of the lipase in the presence of guanidine showed that the inhibition was not just a result of reducing the number of active lipase molecules.

Examination of the amino acid sequences of the lid regions of the lipases from *R. miehei*, porcine pancrease, *H. lanuginosa* and *C. rugosa* showed the presence of an arginine residue only in those lipases that were found to be inhibited by guanidine (Tables 3 and 4). The lack of inhibition by guanidine of some lipases showed that the inhibition was specific and not a general feature of lipases or an artifact of the analyses. The future evaluation of three-dimensional structures of lipases, protein engineering, molecular dynamic simulations and inhibition studies will contribute to the understanding of lipase catalysis.

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Effect of Phosphatidylcholine Structure on the Adenylate Cyclase Activity of a Murine Fibroblast Cell Line

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To determine which structural characteristics of membrane phospholipids influence adenylate cyclase activity, we measured basal and sodium fluoride- or forskolin-stimulated activity in a murine fibroblast cell line, *i.e.*, Balb/c3T3 cells grown in media supplemented with fetal calf serum (FCS), lipid-depleted FCS (LD-FCS) or LD-FCS complexed with different phosphatidylcholine (PC) molecular species. Cells grown in the presence of LD-FCS showed a substantial decrease in their basal and NaF-stimulated adenylate cyclase activities; however, their forskolin-stimulated activity was not altered, suggesting that the enzyme's catalytic site is not affected by changes in membrane lipids. Media supplemented with different LD-FCS/PC complexes were shown to prevent the LD-FCS-mediated reduction of basal and NaF-stimulated adenylate cyclase activity to different extents. Addition of *cis*-9-16:1/*cis*-9-16:1, *cis*-9-18:1/*cis*-9-18:1 or *cis*-9-18:1/*cis*-9,12-18:2 *sn*-glycerophosphocholine (GPC) completely restored adenylate cyclase activity, while *cis*-11-18:1/*cis*-11-18:1 GPC was not effective and only a partial recovery was observed with 16:0/16:0, 16:0/*cis*-9-18:1 and *trans*-9-18:1 GPC. Considering the structural features of these seven PC molecular species, the findings suggest that an optimal lipid environment is conferred to the enzyme by the presence of two *cis* double bonds, each located in Δ9 position of the PC acyl chains. The limited effect of *cis*-9-16:1/*cis*-9-18:1 GPC and *cis*-9-18:1/*cis*-9-16:1 GPC suggests that an equal length of the terminal hydrocarbon chains extending beyond the Δ9 double bonds is also important. Moreover, complete restoration of adenylate cyclase activity in cells exposed to 16:0/*cis*-9,12-18:2 GPC suggests that two *cis*-9,12 double bonds located on the same chain are as effective as two *cis*-9 double bonds each located on two different chains of PC. As the four double bonds of 16:0/*cis*-5,8,11,14-20:4 GPC had no effect, a mere increase in the number of double bonds seems insufficient to build an optimal lipid microenvironment for the enzyme. *Lipids 28,* 727–730 (1993).

Adenylate cyclase [ATP pyrophosphate lyase (cyclizing), EC 4.6.1.1] is a membrane-associated enzyme which, by producing the second messenger cAMP, affects various cellular physiological activities (1). The dependence of adenylate cyclase on lipids has been shown by a number of studies, including dietary manipulation (2–11), treatment with phospholipases (12–17), and incorporation into cell membranes of exogenous fatty acids (18–23), intact phospholipid molecules (24–32) or variations in phospholipid polar head groups (19,20,33). We feel that, under physiological conditions, the activity of adenylate cyclase may well be affected by the phospholipid molecular species present in the membranes (34). This relationship, has only been explored in a few instances (28–32), and only for a limited number of molecular species.

In the present study we determined to what extent phosphatidylcholines (PC) with a defined acyl chain composition affect the reduction of the adenylate cyclase activity which occurs in cell cultures grown in media supplemented with lipid-free serum (22). Adenylate cyclase activity was measured in a murine fibroblast cell line, *i.e.*, Balb/c3T3 cells grown in media supplemented with fetal calf serum (FCS), lipid-depleted FCS (LD-FCS), or in LD-FCS complexed with a series of PC which differed in the length of their acyl chains and in the number, location and configuration of their double bonds. We measured the basal activity of adenylate cyclase as well as the activity stimulated by sodium fluoride or forskolin to explore the G_s protein and the catalytic unit of the enzyme, respectively (35). Using this protocol, we determined which structural features of the phospholipids are important to restore the activities of the regulatory and/or catalytic unit of the adenylate cyclase complex.

MATERIALS AND METHODS

Synthesis of PC. Symmetric PC, dipalmitoyl (16:0/16:0), dipalmitoleoyl (*cis*-9-16:1/*cis*-9-16:1), dioleoyl (*cis*-9-18:1/*cis*-9-18:1), divaccenoyl (*cis*-11-18:1/*cis*-11-18:1) and dielaidoyl (*trans*-9-18:1/*trans*-9-18:1) *sn*-glycero-3-phosphocholines (GPC) were synthesized by reaction of fatty acid anhydrides with the cadmium chloride/GPC complex in the presence of 4-pyrrolidinopyridine as catalyst (36). Asymmetric PC molecules, namely 1-palmitoyl-2-oleoyl (16:0/*cis*-9-18:1), 1-palmitoleoyl-2-oleoyl (*cis*-9-16:1/*cis*-9-18:1), 1-oleoyl-2-palmitoleoyl (*cis*-9-18:1/*cis*-9-16:1), 1-palmitoyl-2-linoleoyl (16:0/*cis*-9,12-18:2) and 1-oleoyl-2-linoleoyl (*cis*-9-18:1/*cis*-9,12-18:2) GPC, were synthesized by submitting the respective symmetric PC (16:0/16:0, *cis*-9-16:1/*cis*-9-16:1, *cis*-9-18:1/*cis*-9-18:1 GPC) to phospholipase A_2 hydrolysis, followed by acylation of the 1-acyl-GPC in position 2 with the appropriate fatty acid anhydrides, using 4-pyrrolidinopyridine as catalyst (36). 1-Palmitoyl-2-arachidonoyl (16:0/*cis*-5,8,11,14-20:4) GPC was supplied by Avanti Polar Lipids (Alabaster, AL). All PC used in this study were free of hydroperoxides and hydrolysis products, as was shown by high-performance liquid chromatographic (HPLC) analysis (37,38).

Preparation of LD-FCS, reconstituted FCS and LD-FCS/PC complexes. LD-FCS was prepared by successive extractions of FCS with an ethanol/diethyl ether (3:1, vol/vol) mixture at −25°C (39). Lipids extracted from FCS were solubilized in a small volume of chloroform and mixed with dry LD-FCS in order to obtain the reconstituted FCS (R-FCS). Individual PC (200 μg per mL of serum) together with cholesterol (100 μg per mL of serum) were also solubilized in a small volume of chloroform and mixed with dry LD-FCS in order to obtain the various LD-FCS/PC complexes. The dry LD-FCS, R-FCS and LD-FCS/PC complexes were solubilized with distilled water under sonication and brought to the original volume of FCS that had been submitted to lipid extraction. The concentrations of each PC molecular species and cholesterol

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Abbreviations: DMEM, Dulbecco's modified Minimal Essential Medium; FCS, fetal calf serum; GPC, *sn*-glycero-3-phosphocholine; HPLC, high-performance liquid chromatography; LD-FCS, lipid-depleted fetal calf serum; PC, phosphatidylcholine; PMV, plasma membrane vesicles; R-FCS, reconstituted fetal calf serum.

complexed with LD-FCS corresponded to the concentrations of the total PC fraction (200 μg per mL) and free cholesterol (100 μg per mL) found in the original FCS (40).

Cells and growth conditions. Balb/c3T3 cells (clone A31) were seeded at 1×10^6 cells per dish (100-mm Falcon tissue culture dishes) and grown at 37°C in a humidified atmosphere of 5% CO_2 in air using Dulbecco's modified Minimal Essential Medium (DMEM) (Grand Island Biological Company, Grand Island, NY) supplemented with 10% FCS (Boehringer Mannheim, Mannheim, Germany). After three days, cell cultures were shifted to DMEM supplemented with 10% LD-FCS, R-FCS or LD-FCS/PC complexes and maintained in this media for 24 h. As shown in a previous study (41), this time period was sufficient to cause changes in the phospholipid fatty acid profiles, which were consistent with the structure of the incorporated PC. Cell layers were then washed with cold 40 mM Tris-HCl buffer, pH 8.3, and scraped from the dish using a rubber policeman. Balb/c3T3 cells cultured for the entire 4-d period in the presence of FCS served as control.

Balb/c 3T3 cells were also exposed for 24 h to media supplemented with LD-FCS complexed with di[1-^{14}C]palmitoyl (16:0/16:0), 1-palmitoyl-2-[1-^{14}C]oleoyl (16:0/*cis*-9-18:1), di[1-^{14}C]oleoyl (*cis*-9-18:1/*cis*-9-18:1) or 1-palmitoyl-2-[1-^{14}C]-linoleoyl (*cis*-9-18:1/*cis*-9,12-18:2) GPC (NEN DuPont, Boston, MA); the specific activities of these radiolabeled PC in the complexes were 2521, 2575, 2698 and 2569 dpm/nmol, respectively. These cultures were used for the preparation of plasma membrane vesicles (PMV) and for the determination of the amounts of the radiolabeled PC which became incorporated into plasma membranes of Balb/c3T3 cells. These amounts served as an indicator of the enrichment of Balb/c3T3 plasma membranes with the various PC used in our experimental protocol.

Incorporation of radiolabeled PC into PMV. Balb/c3T3 cells grown for 24 h in the presence of radiolabeled PC were treated with 25 mM formaldehyde/2 mM dithiothreitol in phosphate-buffered saline to prepare PMV according to Scott *et al.* (42). Measurement of cholesterol (43) and phospholipid (44) content in our preparations of PMV from Balb/c3T3 cells gave a 0.793 cholesterol/phospholipid molar ratio, which is close to the value reported by Scott *et al.*(42). Radiolabeled lipids were extracted from PMV according to Folch *et al.* (45) and fractionated by HPLC following the procedure reported by Schlager and Jordi (38). Chromatography was performed on a 250 × 4.6 mm Silica B5 column mounted in a Perkin-Elmer HPLC apparatus Series 3B (Norwalk, CT), and radioactivities of the various lipid fractions were monitored by using a Flo-One radiochromatographic detector (Radiomatic, Meriden, CT).

Adenylate cyclase assay. Adenylate cyclase activity was determined by measuring the conversion of [α-^{32}P]ATP into cyclic [^{32}P]AMP. The incubation assay was started by adding the cell suspension (22.5–135.0 μg of protein) to the reaction mixture (100 μL final volume) containing 40 mM Tris-HCl (pH 8.3), 2 mM ATP, 2 mM $MgCl_2$, 8 mM theophylline, creatine kinase (6 mg/mL), 5 mM phosphocreatine and [α-^{32}P]ATP (30 Ci/mmol; Amersham, England) ($4–6 \times 10^6$ cpm). Stimulated activity was measured in the presence of 10 mM NaF or 0.01 mM forskolin (Calbiochem, San Diego, CA). The reaction was carried out at 37°C for 15 min, during which time the production of cyclic AMP was linear and proportional to protein concentration. The reaction was terminated by the addition of 100 μL of a solution containing 45 mM ATP, 1.3 mM cyclic AMP and 2% sodium laurylsulfate, and the reaction tubes were placed in a boiling water bath for 3 min until cell proteins were completely solubilized. The reaction mixtures were brought to 1.2 mL with distilled water, and the cyclic [^{32}P]AMP was isolated from the other reaction products by using the chromatographic system described by Salomon (46). The recovery of cyclic [^{32}P]AMP through this chromatographic system ranged from 73 to 86%, as assessed by using a cyclic [^{3}H]AMP internal standard. Proteins were determined by the method of Lowry *et al.* (47) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

As shown in Table 1, the basal adenylate cyclase activity of Balb/c3T3 cells grown in control medium was increased 3.5 and 19 times upon stimulation with NaF or forskolin, respectively. Balb/c3T3 cells grown in a medium supplemented with LD-FCS showed a 40% reduction in adenylate cyclase activity. This effect was also found in a neuroblastoma × glioma hybrid line grown in the presence of lipid-free serum (22), and recalls the reduced adenylate cyclase activity shown in plasma membranes of animals fed an essential fatty acid-deficient diet (2,5). The reduction of the adenylate cyclase activity of cells grown in LD-FCS medium was due only to lipid depletion of the medium, in view of the fact that cells grown in a R-FCS medium showed the same enzymic activity as that found in cells grown in an FCS medium.

The basal adenylate cyclase activity of Balb/c3T3 cells grown in media containing LD-FCS complexed with 16:0/16:0 GPC was higher than that of cells grown in LD-FCS alone, although it did not reach the level found in the cells grown in an FCS medium. A partial restoration of the enzymic activity was also found in Balb/c 3T3 cells grown in the presence of LD-FCS complexed with 16:0/*cis*-9-18:1 GPC.

The adenylate cyclase activity was completely restored in Balb/c3T3 cells grown in the presence of *cis*-9-16:1/*cis*-9-16:1 or *cis*-9-18:1/*cis*-9-18:1 GPC, molecular species characterized by two double bonds, each located at the Δ9 position of the hydrocarbon chain. Divaccenoyl-GPC, with its double bonds at Δ11 positions, was completely ineffective in re-establishing adenylate cyclase activity of Balb/c3T3 cells, which suggests that a specific location of double bonds at Δ9 position is needed in order to bring the adenylate cyclase activity back to normal. Moreover, a *cis* configuration of the Δ9 double bonds is required to achieve normal enzymic activity in view of the lesser effect shown by *trans*-9-18:1/*trans*-9-18:1 GPC. A further requirement appears to be that the terminal hydrocarbon chains extending beyond the Δ9 double bonds be of equal length as growth in the presence of *cis*-9-16:1/*cis*-9-18:1 or *cis*-9-18:1/*cis*-9-16:1 GPC was only partially effective in restoring adenylate cyclase activity.

A complete restoration of adenylate cyclase activity of Balb/c3T3 cells was also obtained by growing the cells in the presence of *cis*-9-18:1/*cis*-9,12-18:2 GPC, which could be explained by the symmetric location of double bonds in Δ9 position of both oleic and linoleic acids. It may also be that linoleic acid, with its double bonds located in Δ9 and Δ12 positions, is sufficient *per se* to restore the enzymic activity. Indeed, 16:0/*cis*-9,12-18:2 GPC produced

TABLE 1

Effect of Different Phosphatidylcholines on the Adenylate Cyclase Activity of Balb/c3T3 Cells[a]

Growth media	Adenylate cyclase activity (pmol of cAMP/min/mg of proteins)								
	Basal			NaF-stimulated			Forskolin-stimulated		
FCS	(10)	23.5 ± 1.7	*100*	(10)	81.9 ± 5.0	*100*	(8)	442.9 ± 37.8	*100*
LD-FCS	(10)	14.5 ± 0.3	*62.0*[b]	(10)	48.3 ± 2.1	*59.0*[b]	(5)	417.8 ± 17.3	*94.0*
R-FCS	(2)	24.4 ± 1.8	*103.8*[c]	(2)	96.2 ± 17.7	*117.4*[c]	(2)	429.6 ± 13.3	*97.0*
LD-FCS+1,2-dipalmitoyl-GPC	(2)	18.2 ± 1.0	*77.5*[b,c]	(2)	70.4 ± 0.8	*86.0*[b,c]	(1)	376.4	*85.0*
LD-FCS+1-palmitoyl-2-oleoyl-GPC	(2)	16.5 ± 0.8	*70.5*[b,c]	(2)	58.9 ± 2.4	*72.0*[b,c]	(2)	389.7 ± 31.0	*88.0*
LD-FCS+1,2-dipalmitoleoyl-GPC	(4)	21.3 ± 1.7	*90.7*[c]	(2)	76.5 ± 2.0	*93.5*[c]	(2)	431.8 ± 42.0	*97.5*
LD-FCS+1,2-dioleoyl-GPC	(6)	21.2 ± 0.5	*90.3*[c]	(6)	79.4 ± 4.0	*97.5*[c]	(3)	413.4 ± 29.4	*93.3*
LD-FCS+1,2-dielaidoyl-GPC	(2)	17.1 ± 0.4	*73.0*[b,c]	(3)	64.4 ± 6.2	*78.6*[b,c]	(2)	374.2 ± 37.6	*84.5*
LD-FCS+1,2-divaccenoyl-GPC	(2)	12.8 ± 3.1	*54.5*[b]	(3)	56.2 ± 9.2	*68.6*[b,c]	(2)	276.8 ± 28.7	*62.5*[b,c]
LD-FCS+1-palmitoleoyl-2-oleoyl-GPC	(2)	17.3 ± 1.5	*74.0*[b,c]	(2)	59.7 ± 6.3	*73.0*[b,c]	(2)	436.2 ± 50.9	*98.5*
LD-FCS+1-oleoyl-2-palmitoleoyl-GPC	(3)	16.4 ± 1.1	*70.0*[b]	(4)	64.9 ± 5.8	*79.2*[b,c]	(2)	398.6 ± 62.0	*90.0*
LD-FCS+1-palmitoyl-2-linoleoyl-GPC	(3)	22.4 ± 2.9	*95.3*[c]	(4)	81.5 ± 9.0	*99.5*[c]	(2)	473.9 ± 8.8	*107.0*
LD-FCS+1-oleoyl-2-linoleoyl-GPC	(2)	27.9 ± 2.6	*119.0*[c]	(2)	89.7 ± 6.1	*109.5*[c]	(1)	500.4	*116.0*
LD-FCS+1-palmitoyl-2-arachidonoyl-GPC	(3)	13.8 ± 1.1	*58.6*[b]	(3)	57.8 ± 1.8	*70.6*[b,c]	(3)	411.9 ± 18.4	*93.0*

[a]Growth media consisted of Dulbecco's Modified Minimal Essential Medium supplemented with 10% fetal calf serum (FCS) 10% lipid-depleted FCS (LD-FCS), 10% reconstituted FCS (R-FCS) or 10% LD-FCS complexed with various phosphatidylcholines. Values are means ± SEM of the number of experiments listed in parentheses. Adenylate cyclase activities of cells grown in manipulated media are also reported as percentages (numbers in italics) of the enzymic activity found in the cells grown in the presence of FCS. GPC, glycero-3-phosphocholine.
[b]Statistically different ($P \leq 0.05$, Student's *t*-test) from the adenylate cyclase activity of cells grown in the presence of FCS.
[c]Statistically different ($P \leq 0.05$, Student's *t*-test) from the adenylate cyclase activity of cells grown in the presence of LD-FCS.

the same adenylate cyclase activity as cells grown in FCS-medium. Adenylate cyclase activity had also been restored to normal in a neuroblastoma × glioma hybrid cell line grown in the presence of lipid-free serum by supplementing growth medium with linoleic acid (22). On the other hand, 16:0/*cis*-5,8,11,14-20:4 GPC was not effective in restoring adenylate cyclase activity. Previous studies (17,21) also had shown that incorporation of arachidonic acid into plasma membranes decreased adenylate cyclase activity. This suggests that a mere increase in the number of double bonds is not sufficient to build an optimal lipid microenvironment for adenylate cyclase.

Adenylate cyclase activity stimulated by NaF was also reduced by growing Balb/c3T3 cells in LD-FCS medium, but this reduction was not observed by growing the cells in R-FCS medium. Moreover, the various PC exhibited almost the same relative stimulatory effects on NaF-stimulated adenylate cyclase activity as they did on the enzyme's basal activity. On the other hand, adenylate cyclase activity stimulated by forskolin was not significantly modified by growth in the presence of LD-FCS or of most LD-FCS/PC complexes, indicating a lack of sensitivity of the catalytic site to changes in the lipid microenvironment. Balb/c3T3 cells grown in the presence of *cis*-11-18:1 *cis*-11-18:1 GPC showed a lower forskolin-stimulated adenylate cyclase activity, possibly due to a specific inhibitory effect of this molecular species on the enzyme.

As shown in Table 2, 16:0/*cis*-9,12-18:2 GPC showed the highest degree of incorporation into PMV, compared to the other radiolabeled PC tested while differences in the incorporation among 16:0/16:0, 16:0/*cis*-9-18:1 and *cis*-9-18:1 GPC failed to reach significance. However, the

TABLE 2

Incorporation of Radiolabeled Phosphatidylcholines into Plasma Membrane Vesicles (PMV) isolated from Balb/c3T3 Cells[a]

PC	No. of exp.	Radioactivity in total lipids (dpm/mg protein)[b]	% of Total radioactivity in PC fraction[c]	Amount of incorporated PC (nmol/mg protein)[d]
1,2-Dipalmitoyl-GPC	3	44682 ± 15021	95.2 ± 1.1	16.9 ± 5.7
1-Palmitoyl-2-oleoyl-GPC	4	25739 ± 6729	91.5 ± 3.7	9.1 ± 2.5
1,2-Dioleoyl-GPC	4	32578 ± 6239	84.5 ± 2.4	10.2 ± 1.9
1-Palmitoyl-2-linoleoyl-GPC	3	55459 ± 7450	97.7 ± 1.2	21.1 ± 2.8[e]

[a]Subconfluent cultures of Balb/c 3T3 cells were grown for 24 h in Dulbecco's Modified Minimal Essential Medium containing 10% LD-FCS complexed with radiolabeled phosphatidylcholine (PC). These cultures were used to prepare PMV by treatment with formaldehyde-dithiothreitol (Ref. 42). Abbreviations as in Table 1.
[b]Radioactivity (means ± SEM) in PMV lipid extracts (Ref. 45).
[c]Percent (means ± SEM) of total lipid radioactivity associated with the PC fraction obtained by high-performance liquid chromatography of total lipids (Ref. 38).
[d]The amount of each radiolabled PC incorporated into PMV was calculated from the radioactivity associated with the PC fraction (taking into consideration the specific activity of the radiolabeled molecular species). Values are means ± SEM.
[e]Statistically different ($P < 0.05$, Student's *t*-test) from the amounts of incorporated 1-palmitoyl-2-oleoyl- and 1,2-dioleoyl-GPC.

degrees of incorporation of the various radiolabeled PC into PMV did not correlate at all with the different effects exerted by these molecular species on adenylate cyclase activity (see Table 1). Thus, the observed effects on adenylate cyclase activity are likely to be related to the structural features of the molecular species studied, and not to the degree of their incorporation into plasma membranes.

Our results support the hypothesis that the activity of the regulatory unit of adenylate cyclase may be controlled by specific molecular species whose number, location and configuration of double bonds in their acyl chains serve to establish an optimal lipid microenvironment.

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Investigations on the Cellular Uptake of Hexadecylphosphocholine

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The uptake of [(9,10)-^{3}H]hexadecylphosphocholine (HePC) in six tumor cell lines was studied. All cell lines incorporated HePC in similar amounts, with the exception of the epidermoid cancer cell line KB, which took up higher amounts of HePC. The uptake of HePC at 37°C was shown to be time and concentration dependent. At 20°C, uptake was drastically reduced and at 4°C it was blocked completely. Binding of HePC, at 4°C, was not saturable at concentrations between 5 μg/mL (11.8 μM) and 100 μg/mL (235.3 μM), indicating that cell surface binding is not receptor-mediated. Furthermore, the effects of inhibitors of endocytosis were investigated. We observed a pronounced inhibitory effect by monensin and cytochalasin B. Colchicine was somewhat less effective whereas chloroquine was almost without effect. From these data we conclude that uptake of HePC is most probably mediated *via* a receptor-independent endocytotic mechanism.

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The ether lipid analogue hexadecylphosphocholine (HePC) is a representative of the group of alkylphosphocholines (1). It is cytotoxic for a variety of tumor cell lines in culture (2,3). Oral treatment of tumor-bearing animals with HePC resulted in significant antitumor activity (4,5); however, the effects were highly specific for certain tumor types. Autochthonous, carcinogen-induced rat tumors as well as some human tumor cell lines transplanted onto nude mice were extremely sensitive whereas classical murine screening models such as P388 and L1210 were completely insensitive (4).

Early clinical trials with a topical application of HePC in cutaneous metastases of patients with mammary carcinomas have already indicated that this compound is also active in human cancer (6,7).

The molecular mechanism of action of HePC is still unknown. Studies on the metabolism of HePC in the human leukemia Raji cell line revealed that HePC is only slowly degraded with more than 90% of the radiolabel still being associated with the parent compound after three days of incubation (2). Generation of toxic metabolites does not seem to be the key event of HePC cytotoxicity since a nondegradable analogue of HePC showed similar antiproliferative activities (8). There are, however, several lines of evidence that suggest that HePC may interfere with signal transduction mechanisms in proliferating cells. In this respect, an inhibition of protein kinase C in bombesin-induced NIH 3T3 fibroblasts and also in phorbol-stimulated HL60 cells has been reported (9,10). In interleukin-2 dependent polymorphonuclear cell cultures, a HePC-induced enhancement of interferon-gamma and an increased expression of interleukin-2 receptors could be demonstrated (11). In U937 cells, HePC stimulates an increase of histone H_1^0 gene expression, which clearly precedes growth retardation (12). In addition it has been demonstrated that low doses of HePC amplify the hematopoietic growth factor dependent proliferation of progenitor cells *in vitro* (13).

A prerequisite for any kind of biological effect of HePC and other ether lysophospholipid analogues, however, is the fact that cells are able to incorporate these substances into their membranes. The aim of this study was to investigate the mechanism of uptake of HePC into human tumor cell lines.

MATERIALS AND METHODS

Materials. HePC was synthesized as described before (14). [9,10-^{3}H]HePC (11.4 Ci/mmol) was a gift of Asta-Pharma AG (Frankfurt, Germany) and was diluted to 60 mCi/mmol prior to purification on preparative thin-layer chromatography (TLC) plates. The cholesterol determination kit, colchicine, monensin, chloroquine, cytochalasin B and desoxyglucose were from Sigma (Munich, Germany). Sodium thiocyanate was from Merck (Darmstadt, Germany). Ficoll-Hypaque was from Biochrom (Berlin, Germany). Neutrophile isolation medium (NIM) was from Packard (Los Alamos Diagnostics, Los Alamos, NM). Sephadex G-15 was from Pharmacia (Freiburg, Germany) and the phosphorus determination kit, according to Eibl and Lands (15), was from Serva (Munich, Germany). All other chemicals were of commercial grade and used without further purification.

Cell lines. All cells were from the American Type Culture Collection (ATCC, Rockville, MD). KB is a human epidermoid cancer cell line; MDA MB 231 is a human mammary carinoma derived cell line. Swiss 3T3 is a murine fibroblast cell line. HL 60, U937 and K 562 are human leukemic cell lines of myeloid origin. The Raji cell line was isolated from a human Burkitt lymphoma (16).

Cell culture. The cells were all maintained in Click's/RPMI 1640 medium supplemented with 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(3-ethanesulfonic acid), pH 7.3, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM L-glutamin and 10% fetal calf serum (FCS). Cell cultures were checked for mycoplasma contamination at regular time intervals. For subcultering or experiments. adherent cells were detached from the culture flasks by 0.025% trypsin in 20 mM EDTA and washed twice in fresh medium. Suspension culture cell lines were centrifuged and resuspended in fresh medium. Cell number and viability was determined in a Neubauer cell counting chamber by trypan blue dye exclusion (17).

Proliferation assay. Cells ($0.5–2 \times 10^6$/well) were incubated in six-well plates (5 mL medium/well) with HePC for 48 h at 37°C and pulsed with [^{3}H]thymidine (2 μCi/mL) for the last 6 h of culture. Aliquots were taken from each well and transferred to a 96-well microtiter plate, which was then processed in a semi-automatic cell harvester (Cambridge Technology, Cambridge, MA). Cells were collected on glass-fiber filters, and the amount of

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Abbreviations: ATP, adenosine triphosphate; FCS, fetal calf serum; HePC, hexadecylphosphocholine; NaSCN, sodium thiocyanate; NIM, neutrophile isolation medium.

[^{3}H]thymidine incorporated was determined by liquid scintillation counting using a Packard model Tricarb counter (Packard Instruments, Frankfurt, Germany). The rest of the cultures was transferred into 15-mL tubes, centrifuged at 400 × *g* for 10 min and resuspended in 1 mL culture medium. Cell number and viability were determined in a Neubauer cell counting chamber by Trypan blue dye exclusion.

[^{3}H]HePC uptake. Cells (0.5 to 4 × 10^6/mL) were incubated at 37°C for 2 h before [^{3}H]HePC (0.1 μCi/mL) was added. Simultaneously, unlabeled HePC was added at the desired concentration. During this preincubation time, cells were either left untreated, treated with endocytotic inhibitors or treated with inhibitors of adenosine triphosphate (ATP) generation. At indicated time intervals, aliquots were taken and washed three times in ten volumes of ice-cold phosphate buffered saline containing 5% FCS. Cell pellets were finally lysed in liquid scintillation fluid, and cell associated radioactivity was measured by liquid scintillation counting.

Phospholipid and cholesterol contents. Cellular lipids were extracted from a known number of cells with hexane/isopropanol (3:2, vol/vol) and dried under nitrogen. The extracts were then redissolved in chloroform/methanol/water (5:10:2, by vol), desalted over a Sephadex G-15 column, and dried again. Lipid phosphorus was then determined by the method of Eibl and Lands (15). Total cholesterol was assayed by an enzymic color reaction according to Sale *et al.* (18).

Preparation of blood leukocytes. Citrated whole blood was layered on top of a Ficoll-Hypaque/NIM (1:4, vol/vol) mixture. After centrifugation at 400 × *g* for 45 min, granulocytes and lymphocytes were collected with a Pasteur pipette from the upper interface and the lower interface, respectively. The granulocyte and the lymphocyte fractions were tested for contaminating cells in an automated cell counter and were found to be more than 90% pure. Both cell fractions were washed three times in fresh medium and incubated at 37°C for 1 h before [^{3}H]HePC and HePC was added. Uptake of HePC was monitored as described for the cultured cells.

RESULTS

Lipid content of the cell lines. The cell lines used in this study showed a large variation in membrane phospholipid and cholesterol content reflecting their different cell size (Table 1). Besides having varying lipid contents, these cell lines also reacted quite differently to HePC. The sensitivity toward HePC in our proliferation assays (Table 2) ranged from 0.5 μg/mL (1.18 μM) HePC for the most sensitive cell line (KB) to above 35 μg/mL (82.4 μM) for K562 cells.

The difference in sensitivity for HePC between the cell lines, however, neither correlated with the phospholipid content nor with the cholesterol content of the cells. Also, no correlation between sensitivity toward HePC and the phospholipid-to-cholesterol ratio was found between different cells.

Time dependence of uptake. The uptake of HePC (10 μg/mL, 23.5 μM) was investigated at 37°C on the cell lines listed in Table 1 and on freshly prepared human granulocytes, lymphocytes and erythrocytes (Fig. 1). To account for cell size differences, uptake of HePC was expressed as nmol per 100 nmol cellular phospholipids. All cells incorporated HePC in a time-dependent manner. With the exception of the KB cell line, uptake was linear with time for the first 2–3 h of incubation. Independent of the

TABLE 1

Cellular Lipid Content[a]

Cell line	Lipid phosphorus (nmol/10^6 cells)	Cholesterol (nmol/10^6 cells)	Ratio[b]
KB	108.8 ± 13.1	16.0 ± 4.8	6.8
MDA MB 231	110.8 ± 11.5	24.4 ± 7.5	4.5
HL 60	40.7 ± 7.1	7.4 ± 3.0	5.5
K 562	65.1 ± 5.2	17.9 ± 2.4	3.6
U 937	38.0 ± 5.5	10.1 ± 1.5	3.7
Raji	35.5 ± 2.0	8.7 ± 1.8	4.1
Swiss 3T3	143.9 ± 14.4	29.5 ± 18.3	4.9

[a]Lipid phosphorus and cholesterol content were determined on lipid extracts of a known number of cells. Data represent the mean ± SD of at least five independent determinations.
[b]The ratio of lipid phosphorus *vs.* cholesterol, calculated from the mean values.

TABLE 2

Sensitivity of the Cell Lines to Hexadecylphosphocholine[a]

Cell line	ID_{50} (a) (μM)	ID_{50} (b) (μM)	LD_{50} (c) (μM)
KB	1.6 ± 0.6	5.4 ± 3.0	3.1 ± 2.3
MDA MB 231	19.1 ± 3.1	50.4 ± 12.9	41.2 ± 6.5
HL 60	6.6 ± 4.5	5.4 ± 3.2	5.2 ± 2.6
K 562	56.7 ± 4.3	66.6 ± 4.5	>85
U 937	5.9 ± 3.0	8.0 ± 0.7	17.6 ± 2.3
Raji	7.1 ± 4.2	16.9 ± 7.2	58.8 ± 6.0
Swiss 3T3	>85	n.d.	>85

[a]ID_{50} values were calculated based on cell count (a) or [^{3}H]-thymidine incorporation data (b). LD_{50} values were calculated from trypan blue dye exclusion asays (c). Data are the mean values ± SD of three to six independent experiments; n.d., not determined.

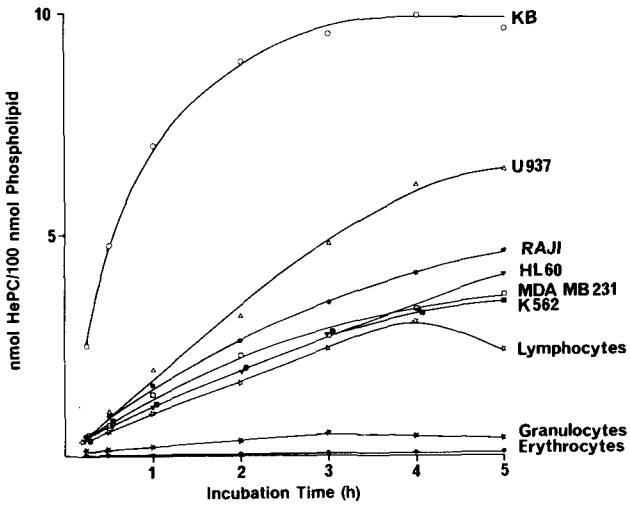

FIG. 1. Time-dependent uptake of hexadecylphosphocholine (HePC) in different cells. The amount of HePC taken up is calculated per 100 nmol cell phospholipid. Incubation conditions are given in Materials and Methods. Data show the mean values of three independent experiments.

number of cells in incubation (0.5 to 4 × 10^6/mL), the rate of uptake of HePC per 100 nmol cellular phospholipids was constant for each cell line tested. However, saturation was not reached even after 5 h of incubation. For KB cells, uptake was very fast and apparently saturation was reached already after 3 h of incubation. A correlation between the uptake rate of HePC and sensitivity for this compound was not observed, since Swiss 3T3 and HL 60, the most resistant and one of the most sensitive cell lines, respectively, took up HePC at similar rates, leading to an almost identical HePC-to-phospholipid ratio.

Concentration dependence of uptake. For subsequent investigations we selected four tumor cell lines: KB, MDA MB 231, HL 60 and K 562. A concentration-dependent uptake of HePC (5–20 μg/mL, 11.8–47.1 μM) at 37°C for the selected cell lines is shown in Figure 2, indicating that the uptake of HePC was linearly dependent on the HePC concentration.

Temperature dependence of uptake. Next we studied the effect of lower temperatures on the uptake of HePC (Fig. 3). In all four cell lines the uptake was strongly inhibited at 20°C. At 4°C no uptake was observed at all.

Furthermore, the release of [^{3}H]HePC from cells preloaded for 1 h at 37°C was also found to be temperature-dependent (data not shown). After 2 h of incubation in HePC-free medium, cells had lost about 30% HePC at 37°C and about 10–15% at 4°C. At the lower temperature, however, an increasing amount of the cells became trypan blue positive after prolonged incubation so that the real amount of HePC release was probably much lower.

Binding at 4°C. To test the possibility of receptor-mediated binding of HePC, the cells were incubated with 1 μCi [^{3}H]HePC and increasing amounts of unlabelled HePCat 4°C. Binding was linear with HePC concentration up to 100 μg/mL (235.3 μM), indicating that HePC binding is probably not receptor-mediated (Fig. 4). The maximum concentration was high enough to lyse the cells after about 3 h of incubation, even in the presence of 10% FCS. KB cells, for instance, were damaged severely as

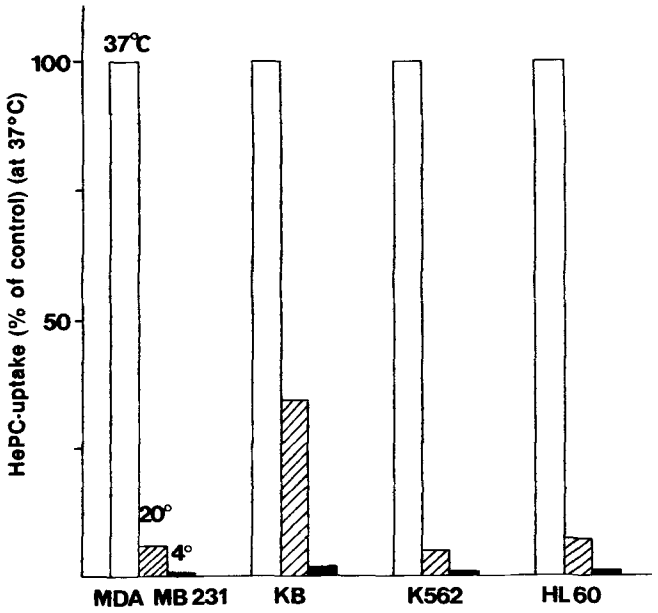

FIG. 3. Influence of low temperature on hexadecylphosphocholine (HePC) incorporation. Cells were incubated at the indicated temperature for one hour before HePC was added. Then incubation was continued for another two hours. After this period the cells were processed by the standard procedures as described in Materials and Methods. Data shown are the mean values of two independent experiments in duplicate.

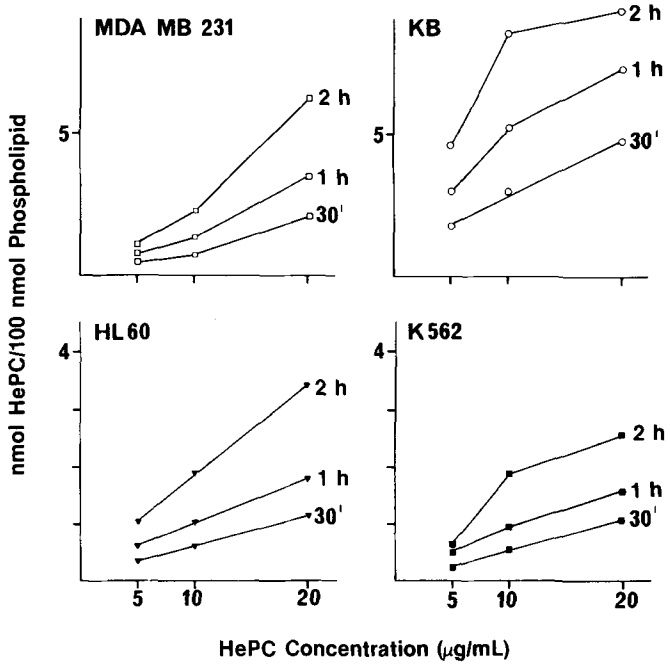

FIG. 2. Concentration-dependent uptake of hexadecylphosphocholine (HePC) in four selected cell lines. The incubation time was 45 min at 37°C. Data shown are the mean values of two independent experiments in duplicate.

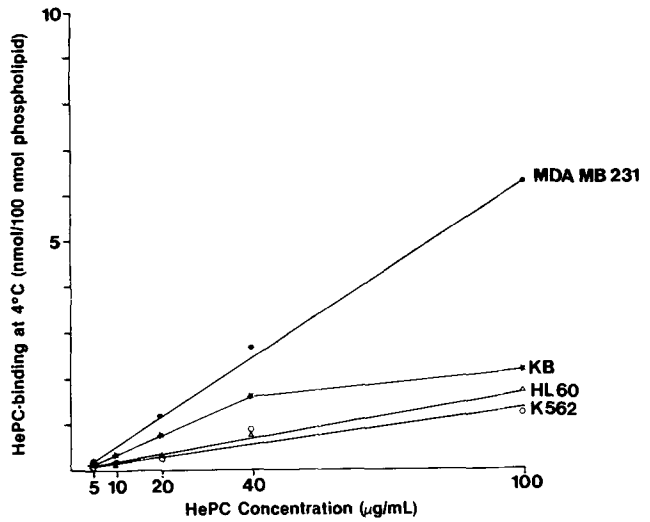

FIG. 4. Binding of hexadecylphosphocholine (HePC) to the extracellular surface of the plasma membrane. Binding studies were performed at 4°C to prevent internalization of HePC. The amount of HePC bound to the extracellular surface was determined after two hours of incubation at the concentration of HePC indicated. Data shown are the mean values of two independent experiments in duplicate.

shown by trypan blue dye exclusion, which leads to a loss of cells in the washing procedure and, consequently, to a decrease in the observed binding of HePC.

Influence of FCS. The effect of FCS in the medium on the incorporation of HePC (5 μg/mL, 11.8 μM) into the tumor cell lines is shown in Figure 5. In the absence of FCS, the cells incorporated substantially higher amounts of HePC showing faster uptake kinetics than in the presence of FCS. We were not able to study uptake kinetics at higher concentrations of HePC or for longer time periods in the absence of FCS because under such conditions the cells were increasingly damaged. In the presence of FCS, however, at 10 μg/mL (23.5 μM) the cells showed no adverse effects for incubation times as long as five hours.

Energy depletion. To investigate whether the uptake of HePC is energy-dependent, the cells were preincubated with sodium thiocyanate (NaSCN, 13.4 μM) and desoxyglucose (62.9 μM), which block oxidative phosphorylation and the glycolytic pathway, respectively (19,20). The compounds alone had little effect on the uptake of HePC (Fig. 6), but a combination of both compounds decreased uptake to between 60 to 80% of controls.

Inhibition of intracellular vesicle transport. The effects of monensin, colchicine, cytochalasin B and chloroquine, well known inhibitors of intracellular transport, are shown in Figure 7. In general, cytochalasin B (41.8 μM) and monensin (29.8 μM) showed rather strong effects, with uptake inhibition up to 50% for monensin and 70% for cytochalasin B. Chloroquine (62.5 μM) and colchicine (3.75 μM) were weaker uptake inhibitors. However, individual cell lines reacted differently to these compounds.

Coprecipitation of HePC with membrane particles. When HL 60, MDA MB 231 or KB cells were loaded for

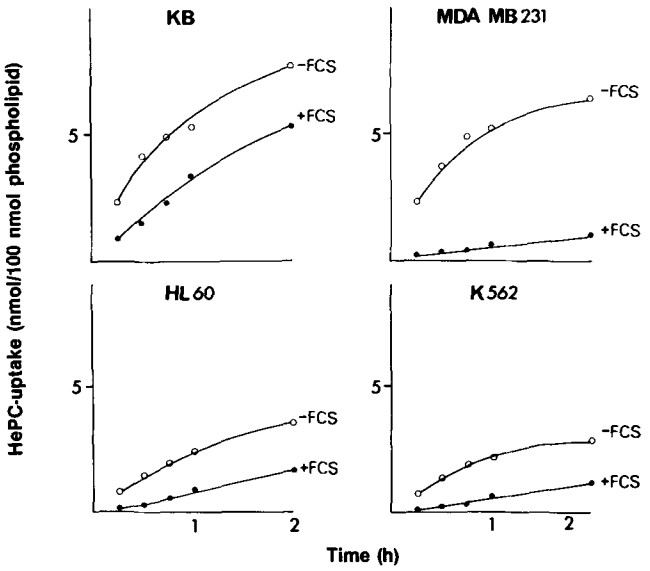

FIG. 5. Influence of fetal calf serum (FCS) on hexadecylphosphocholine (HePC) incorporation. Four selected cell lines were incubated in the presence or absence of 10% FCS, with 5 μg/mL HePC. At indicated time points, aliquots of the cell suspension were taken and incorporation of HePC determined as described in Materials and Methods. Data shown are the mean values of two independent experiments in duplicate.

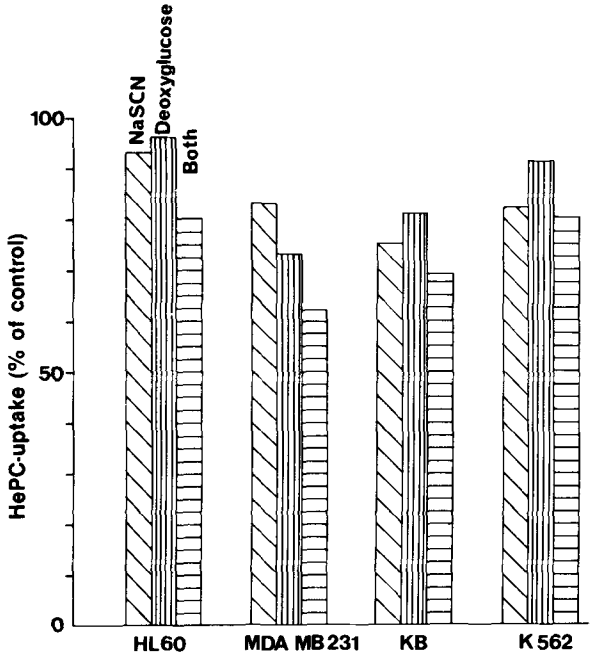

FIG. 6. Effect of energy depletion on hexadecylphosphocholine (HePC) uptake. Cells were preincubated for one hour at 37°C with medium alone (control), sodium thiocyanate (NaSCN) (123.4 μM), deoxyglucose (62.9 μM), or both. Then HePC (10 μg/mL, 13.5 μM) was added, and after two hours of incubation the amount of HePC incorporated in the cells was determined. Data shown are the mean values of three independent experiments.

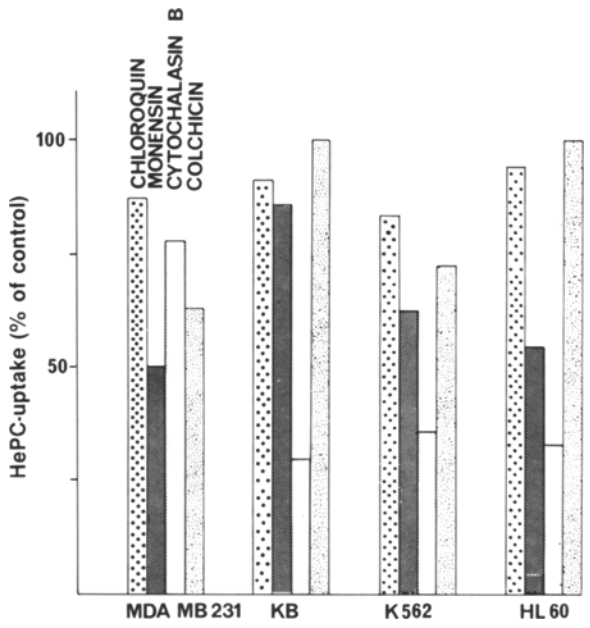

FIG. 7. Effects of inhibitors of intracellular vesicle transport on hexadecylphosphocholine (HePC) incorporation. Cells were preincubated at 37°C for one hour with medium alone (control), monensin (29.8 μM), cytochalasin B (41.8 μM), colchicine 3.75 μM) or chloroquine (62.5 μM). After further incubation with 10 μg/mL (13.5 MM) HePC for one hour, incorporation of HePC in the cells was determined as described above. Data shown are the mean values of two independent experiments in duplicate.

TABLE 3

Coprecipitation of HePC with Membrane Particles of Fractionated Cells

Cell line	Total cell[a] pmol HePC[d]	NaCl-washed[b] pmol HePC[d]	% of total	FCS-washed[c] pmol HePC[d]	% of total
HL 60	360 ± 30	350 ± 35	97	150 ± 20	41
MDA MB 231	600 ± 50	680 ± 70	113	90 ± 20	15
KB	270 ± 30	210 ± 25	77	130 ± 30	48

[a]Hexadecylphosphocholine (HePC) incorporation in fetal calf serum (FCS)-washed whole cells.
[b]HePC incorporation in cell pellets after freezing with distilled water and washing with isotonic NaCl.
[c]HePC incorporation in cell pellets after freezing with distilled water and washing with isotonic NaCl plus 10% FCS.
[d]HePC contents were calculated per 100 nmol phospholipid. Data are mean values ± SD of two independent experiments.

2 h with 10 μg/mL (23.5 μM) of HePC and subsequently washed three times with medium containing 10% FCS, no further removal of HePC from the cells was observed (data not shown). After lysis of these cells by freezing with distilled water, followed by washing with isotonic NaCl, the HePC-to-phospholipid ratio in the cell pellets was very similar to that of the total cells (Table 3). However, when the pellets were washed with NaCl containing 10% FCS, a substantial amount of incorporated HePC could be removed, leading to lower HePC-to-phospholipid ratios for all three cell lines studied.

DISCUSSION

When following the incorporation of lysolipid analogues, such as HePC, which favor various membrane sites of the cells, differences in cell size and membrane lipid content have to be considered. Since HePC is incorporated into cellular membranes, cells with higher amounts of membrane lipids are likely to accommodate more HePC than cells containing less membrane lipid. To account for this possibility, we determined the lipid phosphorus contents of the cells under study and normalized HePC uptake to nmol HePC per 100 nmol cellular phospholipid.

Based on such calculations, we found that under physiological conditions at 37°C, both sensitive and insensitive cell lines took up HePC in similar amounts. Thus, a direct correlation between uptake of HePC and sensitivity toward HePC was not apparent. The epidermoid cancer cell line KB, that is very sensitive to HePC and incorporated about twice the amount as other cells, seemed to have a higher capacity of HePC uptake.

Another parameter that could account for differences in sensitivity is the presence of cholesterol in the cellular membranes, since cholesterol is known to protect against the action of ether lysophospholipids (21) in such a way that even stable liposomes can be prepared from 1-to-1 molar mixture of lysophosphatidylcholine and cholesterol (22). Here again, when comparing different cells, no correlation between the cellular level of cholesterol and HePC sensitivity was found. For instance, HL 60 and Raji cells have a very similar cholesterol content, but showed at least a tenfold difference in LD50. Therefore a simple mechanism for HePC cytotoxicity based on HePC accumulation followed by cell lysis seems unlikely.

For further investigations on the mechanism of HePC uptake, we selected two HePC sensitive cell lines, KB and HL 60, and two resistant lines, MDA MB 231 and K562. In these experiments, we could not detect any significant difference in uptake when comparing monolayers or suspension cultures prepared from the adherent cell lines.

When the cells were incubated with varying concentrations of HePC, uptake was linear with increasing concentrations up to 40 μg/mL (94.1 μM). At 40 μg/mL, the HePC concentration is well above the critical micellar concentration determined for HePC in absence of FCS (*ca.* 8 μM). At 37°C, in the presence of 10% FCS, 42 μg/mL HePC (100 μM) began to induce erythrocyte lysis (not shown), whereas in 40 μg/mL (94.1 μM) the erythrocytes were stable for at least 2 h. Apparently, HePC uptake is not saturable.

For another ether phospholipid, platelet activating factor (PAF), the existence of specific cell membrane receptors has been established (23,24). Therefore, incubations with increasing concentrations of HePC were performed at low temperatures. Under these conditions, uptake of HePC was inhibited (Fig. 3). No saturation of HePC binding sites was attained up to a concentration of 100 μg/mL (235.3 μM), indicating that HePC association with cell membranes is probably not receptor-mediated. The uptake of HePC in the cells is only moderately sensitive to energy depletion. A one-hour preincubation with sodium thiocyanate and deoxyglucose led to a reduction of uptake of about 30%. Since pump proteins normally need ATP as their source of energy (25), or act through a sodium gradient that is maintained by a sodium dependent ATPase (26), we conclude that no HePC specific pump protein is present.

Cytochalasin B, a drug known to desintegrate actin filaments (27), has been reported to be a strong inhibitor for endocytosis (28,29). Also, it drastically decreased the uptake of HePC. Another potent inhibitor of HePC-uptake was monensin. This substance elevates lysosomal and endosomal pH levels thereby leading to a loss of endocytotic activity. It impairs a variety of cellular functions (30,31) and also interferes with the action of certain parasites (32). However, chloroquine, which is also known to elevate the pH in acidic intracellular compartments (33), showed a much weaker effect on HePC uptake. Colchicine, which stabilizes microtubuli and thereby interferes with endocytotic processes (34), was not very active in reducing HePC uptake.

The drastic effects observed with cytochalasin B and monensin strongly point in the direction of a nonspecific endocytosis-dependent mechanism for the uptake of HePC.

Probably, HePC-uptake is based on a membrane renewal process driven by pinocytosis. It has been shown for a variety of cells that high amounts of extracellular fluid or medium and solutes present in the medium are ingested by pinocytosis (35,36), resulting at the same time in substantial exchange of lipids between the plasma membrane and intracellular membranes. Uptake rates of HePC in the presence of serum proteins are about two- to threefold lower than in the absence of serum proteins.

Serum albumin, a protein known to bind lysophosphatidylcholines (37-43), has been reported to also bind ether lysophospholipids such as ET-18-OCH_3 (44) and HePC (45). Serum albumin is also known to extract lysophosphocholines from membranes (39-43). It is therefore also likely to lower the net endocytotic ingestion of HePC by re-extracting this compound from the plasma membrane back into the medium (see Table 3). Thus, serum albumin lowers the effective amount of HePC that is immediately available to the cell *via* membrane recycling.

Our data show, that, with the exception of KB cells, HePC is taken up by the cells to a similar HePC-to-phospholipid ratio in resistant as well as in sensitive cell lines. Uptake is temperature-dependent and can be blocked by inhibitors of endocytosis. In the presence of FCS, HePC-uptake in the cells is lowered, due to binding of HePC to serum albumin (45). Serum albumin is likely to re-extract HePC back from the plasma membrane into solution (Table 3). Since HePC-uptake between 5 μg/mL (11.8 μM) and 40 μg/mL (94.1 μM) is linearly dependent on HePC-concentration, it seems that HePC uptake is not protein-dependent. We therefore suggest that HePC-uptake takes place, after intercalation with the plasma membrane, by a membrane-recycling-dependent endocytotic mechanism. This conclusion is in agreement with data by Bazill and Dexter (46), who found that ET-18-OCH_3 is taken up by an endocytotic mechanism, that can be blocked by the same endocytotic inhibitors as we have used. However, Bazill and Dexter (46) concluded that resistance of cells to ether lysophospholipids is due to a reduced uptake, since resistant cells used in their study were found to incorporate less ET-18-OCH_3. Obviously, HePC must be incorporated into the cells before it can affect cell growth and cell viability, so that inhibition of uptake is one way of protecting cells from the cytotoxic effects of ether lysophospholipids. However, based on our finding, uptake alone can not account for variations in sensitivity for the different cells tested, since the most resistant cell line (3T3) and one of the most sensitive cell lines (HL60) incorporated HePC to reach almost identical HePC-to-phospholipid ratios.

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Systematic Analysis of Glycosphingolipids in the Human Gastrointestinal Tract: Enrichment of Sulfatides with Hydroxylated Longer-Chain Fatty Acids in the Gastric and Duodenal Mucosa

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The composition of the glycosphingolipids of the human gastrointestinal tract was studied. The major neutral glycosphingolipids were ceramide monohexosides (*e.g.*, GalCer, GlcCer), LacCer, Gb_3Cer, Gb_4Cer and more polar ones with more than four sugars, whereas neither Gg_3Cer nor Gg_4Cer were present. The acidic glycosphingolipids consisted of sulfatides and gangliosides such as G_{M3}, G_{M1}, G_{D3} and G_{D1a}. Also a large amount of sulfatides was found in the gastric mucosa and duodenum. The concentrations of sulfatides in the fundic mucosa, antral mucosa and duodenum amounted to 416.0, 933.8 and 682.9 nmol/g of dry weight, respectively, exceeding those in the gastric mucosa and kidney of other mammals. The major molecular species of the sulfatides were identified as I^3SO_3-GalCer with hydroxylated longer-chain fatty acids based on the analyses by gas–liquid chromatography and negative ion fast-atom bombardment mass spectrometry. In contrast, gangliosides in these regions showed a tendency to be lower than sulfatides, and the molar ratios of sulfatides to gangliosides were about 2.0, whereas those in other parts were less than 0.5. A high content of sulfatides in the gastric and duodenal mucosa, where mucosa is easily insulted by acid, pepsin and bile salts, may be closely related to their roles in mucosal protection.

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Gastrointestinal mucosa is a metabolically and mitotically active tissue, with various cells being engaged in digestion and absorption. In particular, gastric mucosa consisting of predominantly parietal cells, chief cells and mucous cells has unique functions, such as hydrochloric acid and pepsin secretion, while protecting the mucosa from such a severe environment. It is therefore of interest to clarify in detail the composition, distribution and metabolism of glycosphingolipids in the gastrointestinal tract. Previous studies on gastrointestinal mucosa have focussed on the characterization of fucolipids with blood group activities (3,4), on the characteristic expression of glyosphingolipids in the intestinal epithelium according to cellular differentiation and development (5-7) and on the structural characterization of sulfoglycosphingolipids in the gastric mucosa (8-11). However, systematic and detailed studies on the glycosphingolipid compositions of the whole gastrointestinal tract from esophagus to colon have not been undertaken.

In a previous paper, we have reported that sulfatides (I^3SO_3-GalCer with hydroxylated longer-chain fatty acids) were enriched in the rabbit gastrointestinal mucosal regions which are continuously exposed to acid and which were more resistant to acid hydrolysis than G_{M3}, the major ganglioside in the mucosal regions (12). Therefore, we have assumed that sulfatides might play a role in mucosal protection from acid environment. To extend our previous findings, we have attempted in the present study to systematically determine the glycosphingolipid composition of the human gastrointestinal tract.

MATERIALS AND METHODS

Tissues. The tissues (esophagus, stomach, duodenum, jejunum, colon and rectum) used in this study were obtained at autopsy from patients who had died of lung cancer, uterine cancer or Parkinson's disease. The whole intact mucosa was separated from the outer fibrosal and muscular layer, rinsed thoroughly with saline at 4°C and kept at −70°C until lipid extraction. Nine mucosal tissue samples were studied (Samples 1-9). Fundus Samples 2 and 3 as well as antrum Samples 4 and 5 were obtained from specific individuals, respectively.

Extraction and separation of glycosphingolipids. Tissues were homogenized in water and lyophilized. Total lipids were extracted from the lyophilized powder successively with chloroform/methanol/water (20:10:1, 10:20:1, 20:10:1, 10:20:1, by vol) and chloroform/methanol (1:1, vol/vol) at 40°C. Aliquots of the combined extracts were used for quantitation of cholesterol and lipid-bound phosphorus, and the remainder of the extracts was applied to a column of DEAE-Sephadex (A-25, acetate form) to separate into neutral and acidic lipid fractions as described previously (13). Neutral glycosphingolipids were prepared from the neutral lipid fraction by acetylation, Florisil column chromatography and deacetylation (14), while acidic glycosphingolipids were prepared from the acidic lipid fraction by mild alkaline hydrolysis. Salts in the neutral and acidic glycosphingolipids were removed by dialysis.

Thin-layer chromatography (TLC). Individual glycosphingolipids were chromatographed on silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (E. Merck Co., Darmstadt, Germany) using the solvent

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Abbreviations: CMH, ceramide monohexoside; FABMS, fast-atom bombardment mass spectrometry; GLC, gas-liquid chromatography; h, hydroxy; HPTLC, high-performance thin-layer chromatography; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; TLC, thin-layer chromatography; GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; Gb_3Cer, globotriaosylceramide; Gb_4Cer, globotetraosylceramide; Gg_3Cer, gangliotriaosylceramide; Gg_4Cer, gangliotetraosylceramide; G_{M3}, II^3NeuAc-LacCer; G_{M2}, II^3NeuAc-Gg_3Cer; G_{M1}, II^3NeuAc-Gg_4Cer; G_{D3}, $II^3(NeuAc)_2$LacCer; G_{D1a}, IV^3NeuAc-,II^3NeuAc-Gg_4Cer; G_{D1b}, $II^3(NeuAc)_2$-Gg_4Cer; G_{T1b}, IV^3NeuAc-,$II^3(NeuAc)_2$-Gg_4Cer. The nomenclature used for gangliosides and other glycosphingolipids follows the system of Svennerholm (Ref. 1) and the recommendation of the IUPAC-IUB Commission (Ref. 2), respectively.

systems A, chloroform/methanol/water (65:35:8, by vol) and B, chloroform/methanol/aqueous 0.5% $CaCl_2$ (55:45:10, by vol) and were detected with orcinol/H_2SO_4 reagent for neutral glycosphingolipids and sulfoglycosphingolipids and with resorcinol/HCl reagent for gangliosides. The mobilities in TLC were compared with those of standard neutral glycosphingolipids (GalCer, LacCer, Gg_3Cer, Gb_4Cer, Gg_4Cer), gangliosides (G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a}, G_{D1b}, G_{T1b}) and sulfatides (I^3SO_3-GalCer).

Quantitative analysis. Cholesterol and lipid-bound phosphorus in the total lipid extracts were determined by gas–liquid chromatography (GLC) using 5α-cholestane as an internal standard (13) and by the method of Bartlett (15) after wet digestion with $HClO_4$ and H_2O_2, respectively. Lipid-bound sialic acid in the acidic glycosphingolipid fraction was measured by the resorcinol/HCl method (16). The individual neutral glycosphingolipids, sulfatides and gangliosides were quantitated densitometrically with a dual wavelength TLC densitometer (CS-9000; Shimadzu Co., Kyoto, Japan) after locating the spots with either orcinol/H_2SO_4 or resorcinol/HCl reagent. Orcinol-positive glycosphingolipids and resorcinol-positive gangliosides were monitored at 420 and 580 nm, respectively, while the control wavelength was set at 710 nm.

TLC-immunostaining. Immunostaining of glycosphingolipids on TLC plates was done as described previously (17). Briefly, after development of the glycosphingolipids on plastic TLC plates (Polygram; Macherey-Nagel, Düren, Germany), the plates were soaked in the blocking buffer [1% polyvinylpyrrolidone (PVP), 1% ovalbumin, 0.02% NaN_3 in phosphate-buffered saline (PBS)] at 37°C for 1 h. Then the developed plates with neutral glycosphingolipids were incubated with rabbit anti-Gg_3Cer (asialo G_{M2}) and anti-Gg_4Cer (asialo G_{M1}) antisera. On the other hand, the developed plates with acidic glycosphingolipids were incubated with cholera toxin (Sigma, St. Louis, MO), which has a strong affinity to G_{M1}, followed by incubation with rabbit anti-cholera toxin antiserum, and with mouse anti-G_{M2} monoclonal antibody, YHD-06, which detects GalNAcβ1-4Gal(3-2αNeuAc or NeuGc)β-structure (18). After the plates were washed five times with the washing buffer (0.1% Tween 20 in PBS) and reacted with blocking buffer at 37°C for 15 min, the antibodies bound to the plates were detected with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse Ig (G + M + A) (Miles Scientific Lab., Naperville, IL) and the enzyme substrate (H_2O_2 and 4-chloro-1-naphthol).

Preparative TLC. Ceramide monohexoside (CMH) and sulfatides were isolated by preparative TLC using solvent systems A and B, respectively. Bands with mobilities similar to CMH and sulfatides, as judged by staining a strip of the TLC plates with orcinol/H_2SO_4 reagent, were scraped from the plates, extracted by sonication in chloroform/methanol/water (10:10:1, by vol), and glycolipids were eluted from the gel by column chromatography.

Carbohydrate and fatty acid analyses. CMH and sulfatides isolated by preparative TLC were subjected to methanolysis with 1 M methanolic HCl at 80°C for 20 h. Fatty acid methyl esters were extracted with *n*-hexane, and the methanolic phase was used for carbohydrate analysis. The fatty acid methyl esters were analyzed by GLC on 3% OV-101 coated on Chrolite using temperature programming from 150 to 250°C at 2°C/min. The peak areas were corrected by comparison with the areas of peaks of an authentic mixture of fatty acid methyl esters, KF and KD (Applied Science Laboratories, State College, PA). The methanolic phase was evaporated to dryness under a flow of nitrogen, and reacetylated with methanol/acetic anhydride (4:1, vol/vol) at room temperature overnight. After evaporation, the carbohydrates in the residues were analyzed by GLC as the *N*-acetyl-*O*-trimethylsilyl derivatives using the same column and temperature programming from 150 to 200°C at 2°C/min.

Structure of sulfatides. Sulfatides were isolated from the acidic glycosphingolipid fraction by Iatrobeads (6RS8060; Iatron Lab., Tokyo, Japan) column chromatography with a linear gradient system of chloroform/methanol/water (70:30:0.5 and 70:30:5, by vol). The homogeneity of the isolated sulfatides was examined by comparing their mobilities on a TLC plate with those of standard sulfoglycosphingolipids, I^3SO_3-GalCer and II^3SO_3-LacCer. The structure of the isolated sulfatides was then analyzed by negative ion fast-atom bombardment mass spectrometry (FABMS) as follows. About 5 μg of the isolated sulfatides was mixed with about 5 μL of triethanolamine, and the resultant mixture was put on a stainless-steel sample holder to be inserted into the FABMS ion source. Analysis was performed by bombardment with a neutral xenon beam with a kinetic energy of 4–6 keV and detection of negative ions with a mass spectrometer (JMS HX-110; JEOL, Tokyo, Japan) equipped with a JMA-3500 computer system (JEOL). Assignment of mass numbers was achieved by comparing the spectrum with that of perfluoroalkyl phosphazine (Ultramark; PCR Inc., Jacksonville, FL).

RESULTS

Neutral glycosphingolipids of the human gastrointestinal tract. A thin-layer chromatogram of the neutral glycosphingolipids from different regions of the human gastrointestinal tract is shown in Figure 1. Neutral glycosphingolipids with mobilities similar to those of GalCer,

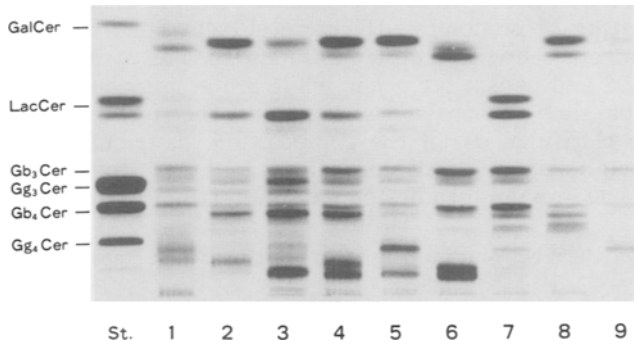

FIG. 1. Thin-layer chromatography of neutral glycosphingolipids from different regions of the human gastrointestinal tract. Lane 1, esophagus (Sample No. 1); lane 2, fundic mucosa (Sample No. 2); lane 3, fundic mucosa (Sample No. 3); lane 4, antral mucosa (Sample No. 4); lane 5, antral mucosa (Sample No. 5); lane 6, duodenum (Sample No. 6); lane 7, jejunum (Sample No. 7); lane 8, colon (Sample No. 8); lane 9, rectum (Sample No. 9); St., standard neutral glycosphingolipids. The amount of lipid applied to each lane corresponded to 2.7 mg dry weight of the respective tissue. Neutral glycosphingolipids were chromatographed on a high-performance thin-layer chromatography plate with solvent system A, and fractions were visualized with orcinol/H_2SO_4 reagent.

LacCer, Gb_3Cer and Gb_4Cer were clearly separated and resolved further into two or three bands presumably due to differences in molecular heterogeneity in their ceramide portion. As described in the result of TLC-immunostaining, neither Gg_3Cer nor Gg_4Cer were present in the normal gastrointestinal mucosa, but several bands corresponding to glycosphingolipids having more than four sugars were seen below Gb_4Cer.

Acidic glycosphingolipids of the human gastrointestinal tract. As clearly shown in Figure 2, sulfatides (I^3SO_3-GalCer) were present in much higher concentration in the gastric mucosa and duodenum than in the other parts of gastrointestinal tract. The mobilities of sulfatides in the antral mucosa and duodenum on a TLC plate were lower than those of standard sulfatides from human brain, indicating that the greater part of these sulfatides contained more hydroxylated, longer-chain fatty acids. The principal gangliosides in the human gastrointestinal tract were G_{M3}, G_{M1}, G_{D3} and G_{D1a}. They amounted for more than 80% of all gangliosides.

Detection of glycosphingolipids by TLC-immunostaining. In order to confirm the structure of glycosphingolipids, TLC-immunostaining with carbohydrate-specific ligands was used. With rabbit anti-Gg_3Cer and anti-Gg_4Cer antisera, neither Gg_3Cer nor Gg_4Cer was detected in the neutral glycosphingolipids from human gastrointestinal mucosa. Binding of cholera toxin to G_{M1} was observed in the acidic glycosphingolipids from all tissues examined. Although the concentration of G_{M1} in the human gastrointestinal mucosa was 30 nmol/g of dry weight as the highest level, which could hardly be detected by conventional TLC using resorcinol/HCl reagent, G_{M1} was easily detected by TLC-immunostaining with cholera toxin (Fig. 3). Gangliosides having the nonreducing carbohydrates of G_{M2} were not detected in the human gastrointestinal mucosa by TLC-immunostaining with mouse anti-G_{M2} monoclonal antibody YHD-06 (18).

Carbohydrate and fatty acid compositions of CMH and sulfatides. The carbohydrate and fatty acid compositions of CMH and of sulfatides are listed in Table 1 and Table 2,

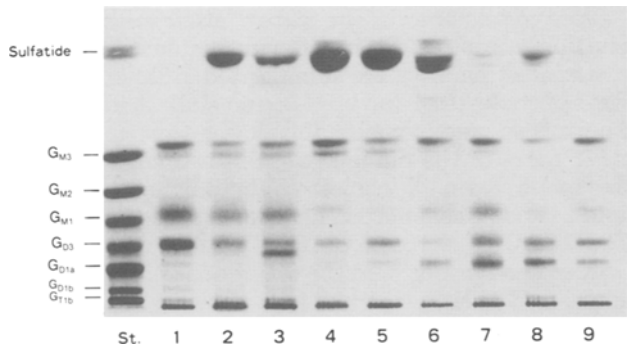

FIG. 2. Thin-layer chromatography of acidic glycosphingolipids from different regions of the human gastrointestinal tract. The lane numbers, 1 to 9, correspond to those in Figure 1. St., standard sulfatides (I^3SO_3-GalCer from human brain) and gangliosides. The amount of lipid in each lane corresponded to about 10 mg dry weight of the respective tissue. Sulfatides and gangliosides were chromatographed on a high-performance thin-layer chromatography plate with solvent system B, and fractions were located with orcinol/H_2SO_4 reagent.

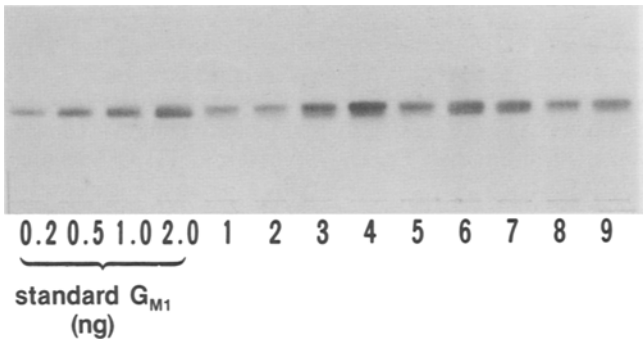

FIG. 3. Thin-layer chromatography (TLC)-immunostaining of ganglioisides from different regions of the human gastrointestinal tract using cholera toxin. Total acidic glycosphingolipids from 0.06–0.07 mg dry weight of the respective tissues and standard G_{M1} (0.2–2.0 ng of sialic acid) were chromatographed on a plastic TLC plate using solvent system B. The plate was tested for cholera toxin binding by TLC-immunostaining. The plate numbers, 1 to 9, correspond to those in Figure 1. A compound without carbohydrate, probably derived from a dialysis bag, was contaminating the region close to G_{M1} and gave a brown spot, not a purple one as for gangliosides, upon resorcinol/HCl staining (see Fig. 2). To assess the correct concentration of G_{M1}, cholera toxin, a specific ligand for G_{M1}, was used. The contaminant explains differences in apparent intensity in the G_{M1} regions in Figure 2 and Figure 3.

TABLE 1

Carbohydrate and Fatty Acid Composition of Ceramide Monohexoside from Different Regions of the Human Gastrointestinal Tract[a]

	Esophagus 1	Fundus 2	Fundus 3	Antrum 4	Antrum 5	Duodenum 6	Jejunum 7	Colon 8	Rectum 9
Molar ratio of carbohydrate									
Glc	1	1	1	1	1	1	1	1	1
Gal	0.48	1.98	1.29	2.18	2.46	0.94	0.88	1.64	1.43
Fatty acid (%)									
Nonhydroxylated									
16–20	28.7	25.3	44.8	29.9	32.4	55.2	44.2	19.0	47.2
22–26	31.7	12.9	13.6	12.6	10.6	10.3	19.8	15.8	14.3
2-Hydroxylated									
16–20	2.9	3.7	9.9	2.7	6.1	4.0	5.9	5.6	2.6
22–26	36.7	58.1	31.7	54.8	50.9	30.5	30.1	59.6	35.9
% of 2-Hydroxylated fatty acids in the total fatty acids	39.6	61.8	41.6	57.5	57.0	34.5	36.0	65.2	38.5

[a]Tissues are identified by sample numbers. For details see Materials and Methods.

TABLE 2

Fatty Acid Composition of Sulfatides from Different Regions of the Human Gastrointestinal Tract[a]

	Esophagus 1	Fundus 2	Fundus 3	Antrum 4	Antrum 5	Duodenum 6	Jejunum 7	Colon 8	Rectum 9
Fatty acid (%)									
Nonhydroxylated									
16-20	72.4	29.4	25.8	29.0	24.5	20.1	60.6	45.1	58.2
22-26	17.5	14.1	13.4	9.3	13.0	11.4	14.5	13.2	15.4
2-Hydroxylated									
16-20	3.1	3.2	3.4	1.4	0.7	0.6	3.0	2.8	4.9
22-26	7.0	53.3	57.4	60.3	61.8	67.9	21.9	38.9	21.5
(28)				(14.1)	(9.6)	(8.4)			
% of 2-Hydroxylated fatty acids									
in the total fatty acids	10.1	56.5	60.8	61.7	62.5	68.5	24.9	41.7	26.4

[a]Tissues are identified by sample numbers. For details see Materials and Methods.

respectively. As shown in Table 1, the concentration of GalCer was higher than that of GlcCer in the gastric mucosa, colon and rectum. In the antral mucosa, the molar ratio of galactose to glucose in CMH was 2-2.5, and the higher proportion of GalCer corresponded to the relatively higher concentration of sulfatides in the antral mucosa that may serve as possible substrate for the synthesis of sulfatides. On the other hand, GlcCer was the predominant CMH in the esophagus, duodenum and jejunum. The fatty acids of CMH and sulfatides in the human gastrointestinal mucosa consisted mostly of nonhydroxylated fatty acids with chain lengths of C_{16} to C_{20} and hydroxylated fatty acids with chain lengths of C_{22} to C_{26}. In particular, more than 60% of the fatty acids of sulfatides in the antral mucosa and duodenum were the hydroxylated longer-chain fatty acids, about 10% of which were 28h:0 (h, hydroxy). Also, there were close similarities between the fatty acid compositions of CMH and of sulfatides in the antral mucosa.

Structure determination of sulfatides. Sulfatides were isolated from the acidic glycosphingolipid fraction of the fundic mucosa (Sample No. 3) by Iatrobeads column chromatography. The isolated sulfatides showed the same mobility on TLC as did I^3SO_3-GalCer from human brain and were directly analyzed by negative ion FABMS. As shown in Figure 4, intense molecular ions, $[M-H]^-$, were observed at *m/z* 778, 794, 878, 890, 906 and 934, corresponding to the sulfatides (I^3SO_3-GalCer) with 4-sphingenine and the fatty acids 16:0, 16h:0, 22h:0, 24:0, 24h:0 and 26h:0, respectively. The relative intensities of the ions were in good agreement with the fatty acid compositions of the sulfatides isolated from the fundic mucosa (Sample No. 3) as determined by GLC (Table 2). In addition, the fragment ions of the terminal sulfate group at *m/z* 97, $[HSO_4]^-$, were clearly identified.

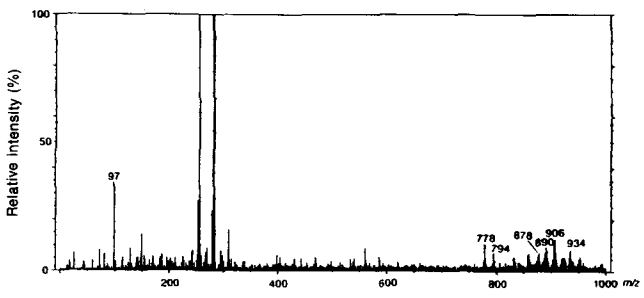

FIG. 4. Negative ion fast-atom bombardment mass spectrometry (FABMS) spectrum of the sulfatides isolated from the human gastric fundic mucosa. About 5 μg of the isolated sulfatides was analyzed by negative ion FABMS with 5 μL of triethanolamine as the matrix solvent.

Lipid composition of the human gastrointestinal tract. The lipid compositions in different regions of the human gastrointestinal tract are summarized in Table 3. The contents of cholesterol and lipid-bound sialic acid in the fundic mucosa were considerably lower than those in the other parts of the human gastrointestinal mucosa, and the molar ratio of cholesterol/lipid-bound sialic acid/lipid-bound phosphorus in the fundic mucosa was 12.0-15.0:0.4-0.7:100.0. CMH (GlcCer, GalCer), LacCer, Gb_3Cer and Gb_4Cer were the major neutral glycosphingolipids, and their concentrations varied in the different regions. Although sulfatides were present throughout the human gastrointestinal tract, the highest content of sulfatides was observed in the gastric mucosa and duodenum. The concentrations of sulfatides in the fundic mucosa, antral mucosa and duodenum amounted to 416.0, 933.8 and 682.9 nmol/g of dry weight, respectively. In contrast, the total amounts of lipid-bound sialic acid in the gastric mucosa and duodenum were lower than those in the other parts, and molar ratios of sulfatides to lipid-bound sialic acid were 1.37-1.97, 2.10-2.33, and 1.95 for the fundic mucosa, antral mucosa and duodenum, respectively, whereas those in the other parts were about 0.5 or below (Table 3).

DISCUSSION

We have previously shown that sulfatides are preferentially enriched in the rabbit gastroduodenal mucosa (12). In the present study, it was clearly demonstrated that human gastric mucosa and duodenum also contains large amounts of sulfatides, exceeding those present in the gastric mucosa of other mammals (8-12,19). More than 60% of the sulfatides in the antral mucosa and duodenum were identified as I^3SO_3-GalCer having hydroxylated longer-chain (C_{22} to C_{28}) fatty acids. α-Hydroxylation of fatty acids in I^3SO_3-GalCer is frequently observed in kidney (20-22) or epithelial cells of mammals (7). For example, contents of I^3SO_3-GalCer with α-hydroxylated

TABLE 3

Lipid Composition in Different Regions of the Human Gastrointestinal Tract[a]

Tissue	Sample number	Cholesterol	Lipid-bound phosphorus	Lipid-bound sialic acid	CMH	G_{M3}	Sulfatide	Moar ratio of sulfatide to gangliosides
		(μmol/g of dry weight)			(nmol/g of dry weight)			
Esophagus	1	20.92	69.80	0.41	454.4	98.4	16.4	0.04
Stomach								
Fundus	2	5.92	49.16	0.21	854.8	92.4	416.0	1.98
Fundus	3	6.09	40.58	0.27	581.8	94.0	370.5	1.37
Antrum	4	19.33	63.06	0.39	1114.6	206.3	820.5	2.10
Antrum	5	21.99	67.78	0.40	1089.9	122.0	933.8	2.33
Duodenum	6	21.17	61.04	0.35	954.4	113.4	682.9	1.95
Jejunum	7	12.98	54.85	0.48	289.4	138.2	218.6	0.46
Colon	8	18.96	75.32	0.47	944.7	81.3	297.3	0.63
Rectum	9	10.78	36.71	0.48	458.0	126.2	153.1	0.32

[a]Fundus Samples 2 and 3, as well as antrum Samples 4 and 5, were obtained from specific individuals. MH, ceramide monohexoside.

longer-chain (C_{22} to C_{26}) fatty acids in the total sulfatides have been reported to be 72% in rat kidney (20), 84% in house musk shrew kidney (21), 37% in human kidney (22), 35% in hog gastric mucosa (8) and 70% in rabbit fundic mucosa (12). It has been proposed that as the fatty acids in the ceramide part of sphingolipids become more hydroxylated and longer-chained, the more stable and impermeable the membranes become (23,24). To protect the cells in the strong acid environment, a more sturdy and acidically charged cell membrane that can resist high concentrations of hydrochloric acid may be required. I^3SO_3-GalCer with hydroxylated longer-chain fatty acids is likely to be a most suitable molecule for that purpose.

In the present study, it was shown that the major neutral glycosphingolipid components in the human gastrointestinal tract are CMH (GalCer, GlcCer), LacCer, Gb_3Cer, Gb_4Cer and probably lacto-series glycosphingolipids including fucolipids. Human gastric mucosa (25) and small intestine (3) are known to be a rich source of fucolipids, which possess antigen activities of the human ABH and Lewis blood group types. On the other hand, the ganglio-series neutral glycosphingolipids (Gg_3Cer and Gg_4Cer) were not present, whereas gangliosides belonging to the ganglio-series were detected in the normal gastrointestinal mucosa. In addition, CMH in the antral mucosa was shown to have a higher proportion of GalCer and a close similarity in fatty acid composition to that of sulfatides, indicating that CMH may serve as the precursor of sulfatides in the antral mucosa. G_{M3}, G_{M1}, G_{D3} and G_{D1a} were the principal gangliosides comprising more than 80% of all gangliosides. We could not find G_{M2} in the normal gastrointestinal mucosa by TLC-immunostaining with anti-G_{M2} monoclonal antibody YHD-06. G_{M2} is considered to be one of the cancer-associated glycosphingolipids in melanoma (26,27), lung carcinoma (28), colonic carcinoma (29) and gastric carcinoma (30). As clearly shown in the result of the binding of cholera toxin to G_{M1} by TLC-immunostaining, G_{M1} is present in the surface of virtually all regions of human gastrointestinal mucosa. *Vibrio cholerae* produces the secretory cholera toxin, which specifically binds to G_{M1} in cell membranes and activates membrane-bound adenylate cyclase through ADP-ribosylation of GTP-binding protein, leading to severe diarrhea (31-33). However, there is no convincing evidence that the cholera toxin has any direct effect on any regions other than the small intestine through G_{M1} receptors. Moreover, little is known about the mechanisms of specific targetting of *V. cholerae* in the small intestine.

It is known that sulfatides exist in the mammalian nervous system, kidney, testis and avian salt gland. A high content of sulfatides in these tissues has been thought to be involved in myelin sheath formation (34), ion transport (35-38) and spermatogenesis (39). Although the presence of sulfatides in the mucosal tissue is well established, their biological function in the gastrointestinal tract has not yet been clarified. However, the preferential enrichment of sulfatides in the gastric mucosa and duodenum, which are continuously exposed to aggressive factors, such as acid, pepsin and bile salts, strongly indicates that sulfatides play an important role in mucosal protection. In addition, in our preliminary study of the effect of sulfatides against experimental ulcers in rats, sulfatides showed antiulcer activity (detailed data to be published elsewhere). Conversely, it has been recently reported that sulfated glycolipids may serve as the mucosal receptor for colonization of *Helicobacter pylori* (40,41), which colonizes only in the gastric epithelium and is now considered to be associated with gastritis and peptic ulcer (42-44). In our experiments, viable *H. pylori* showed a strong binding to sulfatides by TLC-immunostaining with rabbit anti-*H. pylori* antiserum (45). Sulfatides in the gastric mucosa may serve as receptor for the attachment and colonization of *H. pylori*. However, it is still unclear why *H. pylori* has not yet been detected in the duodenum, which also contains a large amount of sulfatides. Further studies on the cellular localization and on the conformation of sulfatides are needed to elucidate the mechanisms of specific attachment and colonization of *H. pylori* in the gastric epithelium.

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Fish Erythrocytes as a Tool to Study Temperature-Induced Responses in Plasma Membranes

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The dorsal aorta of carp (*Cyprinus carpio* L.) was cannulated, and the fish were kept in thermostated aquaria at 5°C for 24 h. The water temperature was then gradually increased to 25°C at a rate of 0.5°C/h, and then decreased to 5°C at the same rate. Blood was withdrawn at five-degree intervals to determine the fluidity of erythrocyte plasma membranes upon *ex vivo* incorporation of the fluorescent dye, 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid. Steady-state anisotropy (R_{ss}) of the plasma membranes increased and decreased with the increase and decrease in water temperature, respectively. The sterol-to-phospholipid ratio remained unchanged throughout the thermal shifts. The fatty acid compositions of the total phospholipids, of phosphatidylcholine and of phosphatidylethanolamine remained virtually unchanged, except for the level of arachidonic acid, which increased in erythrocytes from fish at the higher temperature (25°C). The molecular species compositions of phosphatidylcholines and phosphatidylethanolamines also remained unaffected throughout the thermal shifts. The erythrocyte plasma membranes were more responsive to temperature shifts *in vivo* than *in vitro* when percent efficacy was compared. Thus, factors other than lipid changes are conceivably involved in the adaptation of erythrocyte plasma membranes to short-term thermal changes.

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An inherent property of cells is their ability to maintain the structural and functional integrity of their membranes under changing environmental conditions. Temperature is one of the major external factors influencing membranes in poikilothermic animals. Changes in the composition and physical state of the membranes in response to changes in the prevailing ambient temperature have been observed in most cases studied so far (1,2). Restructuring of existing phospholipids, activation of different desaturases and adjustment of deacylation/reacylation reactions are examples of changes that occur in membranes in response to thermal shifts (3-8). Conventionally, such responses have been studied by disturbing the *status quo* of an experimental specimen and by looking for corrective responses in membranes or membrane lipids in the acclimated state. However, the mode of control of the physical state of membranes under conditions such as those often faced by poikilotherms, *i.e.,* the problem of maintaining function despite fluctuations in ambient temperature, is not well understood.

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Abbreviations:DPH-PA, 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

Most approaches involving the use of higher poikilotherms as experimental models do not allow us to follow the response to external variations within a single specimen. Moreover the specimens usually need to be sacrificed to gather response information. Thus, several specimens are usually required for such studies, and the response observed needs to be measured against variations between specimens.

Fish erythrocytes can be used to study adaptive responses to thermal changes at the membrane level within short time spans as these cells are nucleated and express many functions as do somatic cells. The advantage of studying erythrocytes over isolated membranes from other tissues or cells is the ease of their preparation. Furthermore, a recently introduced fluorescence label 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid (DPH-PA), permits the study of the physical properties of plasma membranes *in situ* shortly after collecting the cells (9). The present study thus provides evidence that carp erythrocytes, obtained from a single specimen in which the dorsal aorta was cannulated, adjust the state and order of their plasma membranes, but not their lipid composition, in response to changes in environmental temperature.

MATERIALS AND METHODS

Experimental design. Male winter-acclimated carp (0.50-0.75 kg), used in these experiments, were obtained from the Tisza Fish Farm (Szeged, Hungary; November 1991-January 1992) at water temperatures of 3-7°C. The fish were kept in thermostated aquaria in the laboratory at 5°C for 3 d for equilibration before the experiments were carried out. Preliminary experiments had indicated that 24 h would be adequate for temperature equilibration for carp of this weight. The fish were anesthetized with MS 222 (10 mg/L) before cannulation of the dorsal aorta by the method of Hughes *et al.* (10). Following cannulation, the fish were kept separately in appropriate compartments for 24 h before starting the experiments. The compartments (two in each experiment, each 3.5 L capacity) were connected to a thermostated water reservoir, from which water was circulated. The water temperature was raised by 0.5°C/h from 5 to 25°C in five-degree steps and then decreased to 5°C again at the same rate. After each step (up-shift or down-shift), the temperature was held for 24 h before sampling. Sufficient blood (50-100 μL) was withdrawn through the cannula (the outlet was otherwise closed) into an Na-heparinized syringe at each temperature (5, 10, 15, 20 and 25°C). For lipid analyses on erthrocytes, 1 mL of blood was withdrawn from separate fish on each occasion.

Labeling of the erythrocytes. To ensure consistency of fluorescence measurements, the optical density of the cell suspension was adjusted to 0.05 absorbance units at 366 nm (the excitation wavelength of DPH-PA) to standardize light scattering of the cells. For cell labeling, 5 μL

of 1×10^{-3} M DPH-PA (Molecular Probes Inc., Eugene, OR) in tetrahydrofuran was added to the cell suspensions in an isotonic buffer solution at pH 7.4 in a 4-mL quartz cell (the lipid-to-probe ratio was 200:1). Labeling was complete within 5 min, as measured by following changes in fluorescence intensity. Washing of the erythrocytes took 10 min, and labeling was complete within 5 min (11).

Fluorescence anisotropy measurements. Fluorescence anisotropy measurements were carried out with a computer-controlled thermostated Hitachi MPF-2A (Hitachi Ltd., Tokyo, Japan) spectrophotofluorimeter. The sample was excited with vertically polarized light at 366 nm at a slit width of 6 nm, and vertically (I_{vv}) and horizontally (I_{vh}) polarized emitted light was read at 430 nm at a slit width of 12 nm. Anisotropy values given are the average of 10 measurements. The standard error of individual determinations was always less than 0.005.

Steady-state fluorescence anisotropy was calculated according to the formula $R_{ss} = I_{(vv)} - Z \cdot I_{(vh)} + 2\,Z \cdot I_{(vh)}$, where $Z = 0.92$, calculated based on the formula $Z = I_{(vh)}/I_{(hh)}$. To correct the fluorescence intensity and R_{ss} for light scattering, measurements were also made on unlabeled samples under identical conditions. The measurements were done according to Kuhry *et al.* (12), who had shown that both the fluorescence intensity and (R_{ss}) are affected by scatter:

$$I_{(measured)} = I_{(fluorescence)} + I_{(scatter)} \quad [1]$$

$$R_{s\,(measured)} = f\,R_{s\,(fluo)} + (1 - f) \cdot R_{s\,(scatter)} \quad [2]$$

where f (a balanced fluorescence intensity factor) = $I_{fluo}/I_{fluo} + I_{scatter}$.

Extraction and analysis of lipids. Lipids were extracted with isopropanol/chloroform (11:1, vol/vol) as recommended by Freyburger *et al.* (13). The extracted lipids were dissolved in benzene containing 0.02% butylated hydroxytoluene as an antioxidant, and stored at −20°C until assayed. Phospholipids were separated by silicic acid column chromatography, using chloroform to elute neutral lipids and methanol to elute phospholipids. Phospholipids were subfractionated by thin-layer chromatography (TLC) according to Fine and Sprecher (14) using pre-coated G-60 silica gel plates (E. Merck, Darmstadt, Germany). Phospholipids were transmethylated under nitrogen with methanol containing 5% hydrochloric acid at 80°C for 2.5 h. Fatty acids were separated on 10% FFAP on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA) using a 2-m column (2 mm i.d.). A Hitachi 263-80 (Hitachi Ltd.) gas–liquid chromatograph (GLC) connected to a Hitachi 263-80 data processor was used for analysis. For measuring the sterol-to-phospholipid ratio, total lipids were separated on TLC plates using petroleum ether/diethyl ether/acetic acid (75:15:1, by vol) as solvent. Phospholipids which did not move from the point of sample application were isolated for quantitative determination of phosphorus according to Rouser *et al.* (15). Free sterols were extracted with diethyl ether and quantitated by gas chromatography using a 3% SP 2250 on 100–200 mesh Supelcoport (Supelco) column as stationary phase and 5 α-cholestane as internal standard. Aliquots of the total lipid extracts were saponified with 0.1 N KOH in ethanol, and nonsaponifiable residues were separated by GLC as described earlier. Phospholipid phosphorus was also determined on the same aliquot.

Molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were analyzed according to Takamura *et al.* (16), by high-performance liquid chromatography (HPLC) (Waters Associates, Milford, MA; Model 440) and detected at 254 nm; the instrument was connected to an integrator (Hitachi 263-80). The dinitrobenzoyl derivatives of the diacylglycerols obtained by phospholipase C (Sigma Chemicals, St. Louis, MO) hydrolysis were separated on a Nucleosyl C-18 (5 μm, 4 mm, i.d. × 250 mm) column with acetonitrile/2-propanol (80:20, vol/vol) of HPLC grade (Carlo Erba, Milan, Italy) used isocratically as mobile phase (flow rate of 1.0 mL/min). Peaks were identified by comparison with those of derivatives of authentic 1,2-diacylglycerol standards and also on the basis of their relative elution times (17); 16:0/22:6 and 18:0/22:6 were obtained from Avanti Polar Lipids (Alabaster, AL), and 16:0/20:4, 18:0/20:4 and 16:0/18:1 were from Sigma; 16:0/22:6 was used as internal standard.

RESULTS

Due to its anionic nature (9), DPH-PA partitions into the outer leaflet of the erythrocyte plasma membrane, which is rich in positively charged phospholipids (18). DPH-PA thus can show changes in the physical state of the plasma membrane due to external stimuli. Dilution of labeled erythrocytes with buffer without DPH-PA leads to the almost complete and rapid efflux of DPH-PA, consistent with the fact that DPH-PA labels only the outer leaflet of plasma membranes (19). In the present study, fish acclimated to a low environmental temperature (5°C) were subjected to controlled up-shifts and down-shifts of ambient water temperature, and changes in fluorescence anisotropy of the probe incorporated into the erythrocyte plasma membranes were followed. The heating and cooling rates were set to mimic, to some extent, the diurnal variations which these fish experience under natural conditions.

Figure 1 reveals that the fluorescence anisotropy of the DPH-PA probe in the erythrocyte plasma membranes *in vivo* varied in a continuous fashion with the change in

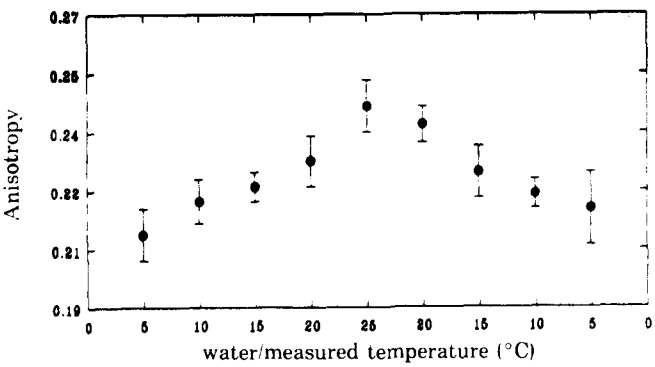

FIG. 1. Changes in 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid fluorescence anisotropy of fish (*Cyprinus carpio* L.) erythrocytes in response to temperature shifts under *in vivo* conditions. Readings were made at the actual (incubation) body temperature (means ± SD of eight experiments).

temperature. The cells responded to an increase in temperature with increased rigidity of their membranes (increase in R_{ss}) and *vice versa*. This raises the possibility that the cells of higher poikilotherms, such as fish, continuously adjust to changes of environmental/body temperature in terms of the physical state of their membranes. Wodtke and Cossins (20) recently reported that carp liver microsomes also adjust their microviscosity according to the actual water/body temperature.

Figure 2 shows a comparison of *in vitro* and *in vivo* experiments, which illustrates that the red blood cells responded *in vitro* in the same way as they did *in vivo*. Figure 2 also shows that, despite these similarities in the response of erythrocytes to temperature up-shifts and down-shifts, the response was always greater *in vivo* than *in vitro*.

Phospholipid analyses showed that there were no significant differences in phospholipid class composition (data not shown). Table 1 gives the fatty acid composition, the total saturated fatty acids and the saturated-to-unsaturated fatty acid ratios of total phospholipids and of PC and PE, and the sterol-to-phospholipid ratios of the erythrocytes at 5 and 25 °C (temperature up-shifted fish). The data show that there was no marked change in the fatty acid composition except for the elevated level of arachidonic acid (20:4) in the temperature up-shifted fish. There also was no change in the saturated-to-unsaturated ratio. The decrease in unsaturated fatty acids and the higher saturated-to-unsaturated fatty acid ratio in the total phospholipids in the erythrocytes before the temperature up-shift may be due in part to the elevated level of myristic acid (14:0). If 14:0 is omitted from the calculations, the ratio decreases from 0.57 to 0.31, which is the same as that for temperature up-shifted cells calculated in the same way. The sterol-to-phospholipid ratio, which is known to affect the physical state of membranes (21–23), also remained unchanged (Table 1).

Table 2 lists the major molecular species of PCs and PEs. Although retailoring of these phospholipids during thermal acclimatization has been demonstrated in unicellular organisms (3,4) and in fish (5), carp erythrocytes did not show such a response during the temperature up-shifts (Table 2) or down-shifts (data not shown). There were only minor changes in the levels of 16:0/20:4, 18:0/22:6 and

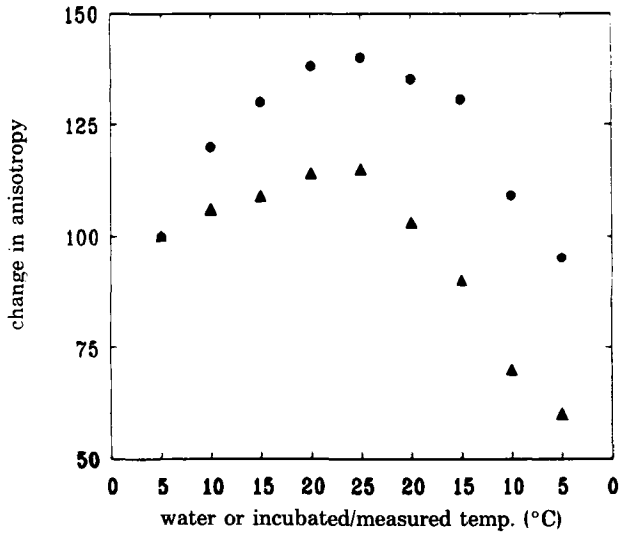

FIG. 2. Comparison of changes in 3-[*p*-(6-phenyl-1,3,5-hexatrienyl)-phenyl]propionic acid anisotropy of fish (*Cyprinus carpio* L.) erythrocytes in relation to temperature shifts under *in vivo* (●—●) and *in vitro* (▲—▲) conditions. In the *in vitro* experiments, erythrocytes were incubated as described in Reference 11. Readings were made at the incubation temperature of the blood/fish in both the cases (actual temperature). Anisotropy values at 25°C were 0.207 ± 0.006 SD in *in vitro* and 0.245 ± 0.007 SD in *in vivo* experiments (means ± SD of eight experiments).

TABLE 1

Fatty Acid Compositions of Total Phospholipids (TPL) and of Phosphatidylcholines (PC) and Phosphatidylethanolamines (PE) of Fish Erythrocytes Before and After Temperature Shift *in Vivo*[a]

	Total phospholipid		PC		PE	
Fatty acid	5°C	5–25°C	5°C	5–25°C	5°C	5–25°C
14:0	11.8	5.6	—	1.0	—	0.5
16:0	14.0	13.8	28.4	26.4	10.5	8.7
16:1n-7	3.1	2.9	2.4	7.8	2.9	2.8
18:0	10.1	10.0	4.4	3.3	5.4	4.4
18:1n-9	13.5	12.9	12.0	13.6	14.5	12.0
18:2n-6	2.1	2.2	1.9	3.0	2.3	2.1
18:3n-3	trace	trace	trace	trace	trace	trace
20:4n-6	20.7	28.5	24.7	28.3	30.5	35.9
20:4n-3	1.1	0.3	0.5	trace	0.6	0.4
20:5n-3	4.2	3.8	5.2	3.0	6.4	5.1
22:4n-6	1.5	3.1	trace	0.7	0.8	2.3
22:5n-6	1.2	2.8	1.2	1.1	1.5	2.6
22:5n-3	3.9	3.7	4.4	0.7	5.4	4.3
22:6n-3	14.1	15.4	14.9	16.5	18.4	18.1
Saturated	35.9	29.4	32.8	30.7	15.9	13.6
Sat/unsat	0.55	0.39	0.49	0.41	0.19	0.15
Sterol/TPL	0.71	0.66	—	—	—	—

[a]n = 8; traces are ≤0.1% of the total.

TABLE 2

Selected Molecular Species from Phosphatidylcholines (PC) and Phosphatidylethanolamines (PE) from Carp Erythrocytes Before and After Temperature Shift from 5 to 25°C *in Vivo*[a]

Molecular species	PC (% of total)		PE (% of total)	
	5°C	5–25°C	5°C	5–25°C
22:6/22:6	0.9	0.8	0.7	0.8
16:1/22:6[b]	3.1	2.6	6.3	2.7
18:1/20:5	trace	trace	1.6	0.4
18:1/22:6[c]	3.1	3.6	6.9	9.8
16:0/22:6	9.3	10.1	12.1	11.1
18:1/16:1	trace	trace	1.3	5.0
16:0/16:1	1.9	1.0	2.0	2.2
16:0/20:4	17.4	19.5	14.8	11.4
18:0/22:6[d]	14.3	12.1	14.3	11.6
18:0/20:4	2.5	2.7	2.8	2.5
18:1/18:1	2.7	2.4	—	0.8
16:0/18:1	35.6	31.8	35.1	29.8
16:0/16:0	trace	trace	trace	trace
Unidentified	9.2	13.4	2.1	11.9

[a]n = 8; traces are ≤0.1% of the total.
[b]Co-eluting with 22:5/22:5.
[c]Co-eluting with 16:0/20:5.
[d]Co-eluting with 16:0/18:2.

16:0/18:1. Our observations thus suggest that the physical properties of fish erythrocyte plasma membranes can vary in response to changes in environmental temperature without changes in lipid composition or sterol-to-phospholipid ratio.

DISCUSSION

The physical state of biomembranes has been shown to depend on their lipid constituents, and temperature-induced changes in membrane fluidity would be expected to be reflected in changes in membrane lipid composition. In the present study, the total phospholipids of the entire erythrocyte were considered representative of those of the average cell membrane, although individual membranes may differ in respect to their lipid components and fluidity, as has been demonstrated for fish liver and muscle (24,25). An inverse relationship between fatty acid unsaturation and temperature has repeatedly been observed (6,26-28). However, our data presented in Table 1 show that under our experimental conditions, carp erythrocytes did not change their saturated-to-unsaturated fatty acid ratio. The sterol-to-phospholipid ratio, which is also known to contribute to the physical state of biomembranes (22,23), has been shown to decrease in fish erythrocytes (21) and mitochondria (21) upon long-term adaptation to lower temperatures. However, in the present study, this ratio remained unchanged irrespective of whether this ratio was expressed in terms of the ratio of free sterols (Table 1) or of total sterols-to-phospholipids (data not shown). It appears, therefore, that these ratios would not play a major role in the short-term acclimatization of fish to temperature. As no differences in fatty acid, phospholipid polar headgroup or molecular species composition were observed, it appears that phospholipid anisotropy does not respond in the same way as it does in intact cells, as we have earlier demonstrated previously (11).

To gain further insight into the nature of the observed control, erythrocytes collected from cold-acclimated fish were also incubated under *in vitro* conditions. In the latter experiments, the whole blood was incubated instead of isolated erythrocytes. As in the *in vivo* experiments, the incubation temperature *in vitro* was also raised from 5 to 25°C and again decreased to 5°C at a rate of 0.5°C/h. The temperature-induced changes in fluorescence anisotropy *in vitro* and *in vivo* (Fig. 2) indicated that the cells were more responsive to temperature changes *in vivo*, although the response was also evident under *in vitro* conditions. It can be speculated that some component of the blood plasma that may exchange with the outer erythrocyte membrane in response to variations in temperature, or some other factors not present in the *in vitro* system, may be involved in the control process. Because blood glucose remained at normal levels throughout the experiment (data not shown), activation of the adrenal gland can be ruled out. Besides catecholamines and other hormones, such as glucocorticoids, adrenocorticotrophic hormone, glucagon and insulin have also been shown to inhibit Δ5 desaturase (29) activity. Thus, their putative effect in temperature up-shifted fish would not need to be taken into consideration either. Further investigations are needed to determine how such a sensitive control of the physical state of the erythrocyte membrane can occur in response to short-term temperature changes in carp.

ACKNOWLEDGMENTS

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Kinetic Study of the Prooxidant Effect of Tocopherol. Hydrogen Abstraction from Lipid Hydroperoxides by Tocopheroxyls in Solution

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A kinetic study of the prooxidant effect of vitamin E (tocopherol, TocH) has been carried out. The rates of hydrogen abstraction (k_{-1}) from methyl linoleate hydroperoxide (ML-OOH) by α-tocopheroxyl (α-Toc•) (1) and eight types of alkyl substituted Toc• radicals (2-9) in benzene solution have been determined spectrophotometrically. The results show that the rate constants decrease as the total electron-donating capacity of the alkyl substituents on the aromatic ring of Toc• increases. The k_{-1} value ($5.0 \times 10^{-1}M^{-1}s^{-1}$) obtained for α-Toc• (1) was found to be about seven orders of magnitude lower than the k_1 value ($3.2 \times 10^{6}M^{-1}s^{-1}$) for the reaction of α-TocH with peroxyl radical, which is well known as the usual radical-scavenging reaction of α-TocH. The above reaction rates (k_{-1}) obtained were compared with those (k_3) of methyl linoleate with Toc• (1-9) in benzene solution. The rates (k_{-1}) were found to be about six times larger than those (k_3) of the corresponding Toc•. The results suggest that both reactions may relate to the prooxidant effect of α-TocH at high concentrations in foods and oils. The effect of the phytyl side chain on the reaction rate of Toc• in micellar dispersions has also been studied. We have measured the rate constant, k_{-1}, for the reaction of phosphatidylcholine hydroperoxide with a Toc• radical in benzene, *tert*-butanol and in Triton X-100 micellar dispersions, and compared the observed k_{-1} values with the corresponding values for ML-OOH.

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The role of vitamin E (α-, β-, γ- and δ-tocopherols) as an important biological antioxidant has been well recognized in recent years. The antioxidant properties of tocopherols (TocH) have been ascribed to hydrogen abstraction from the OH group in TocH by a peroxyl radical (LOO•). The hydrogen abstraction produces a tocopheroxyl radical (Toc•), which combines with another LOO• (Reactions [1] and [2]) (Refs. 1,2).

$$LOO^{\bullet} + TocH \xrightarrow{k_1} LOOH + Toc^{\bullet} \quad [1]$$

$$LOO^{\bullet} + Toc^{\bullet} \xrightarrow{k_2} LOO\text{-}Toc \text{ (nonradical product)} \quad [2]$$

In recent years, several investigators demonstrated that α-TocH at high concentrations acts as a prooxidant during the autoxidation of polyunsaturated fatty acids (LH) in aqueous medium and in bulk phase (3-9). This prooxidant effect of α-TocH leads to an increase of the level of hydroperoxides (LOOH) with conjugated diene structure. Loury *et al.* (3) and Terao and Matsushita (9) have proposed that Toc• radicals participate in this prooxidant effect through 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 Reaction [3] is the chain transfer reaction and Reaction [4] is the reverse of Reaction [1]. A more complex reaction scheme, including a great number of elementary reaction steps, has been proposed by Rousseau-Richard *et al.* (10,11). However, so far the kinetics of Reactions [3] and [4] related to the prooxidant effect of vitamin E have not been studied. The reactions are considered important to the understanding of the prooxidant properties of vitamin E.

We have recently reported the second-order rate constant, k_3, for the reaction of LH esters (ethyl linoleate, ethyl linolenate, ethyl arachidonate and *all cis*-4,7,10,13,16,19-docosahexaenoic acid ethyl ester) with Toc• (5,7-diisopropyltocopheroxyl 5) (12,13). The rate constant, k_3, obtained was 1.82 to $9.05 \times 10^{-2}M^{-1}s^{-1}$ in benzene at 25°C. The rate of k_3 is about 7 to 8 orders of magnitude lower than k_1 for Reaction [1] of α-TocH with LOO• (1). Furthermore, we recently succeeded in measuring the rate constant (k_{-1}) for the reaction of 5,7-diisopropyltocopheroxyl radical 5 with *n*-, *sec*- and *tert*-butyl hydroperoxides used as lipid hydroperoxide model systems (14). The observed rate, k_{-1}, was 1.34 to $3.65 \times 10^{-1}M^{-1}s^{-1}$ in benzene solution at 25°C. The values of k_{-1} are only about one order of magnitude higher than those of k_3.

In the present work, we have determined the second-order rate constant, k_{-1}, for the reactions of methyl linoleate hydroperoxide (ML-OOH) with nine types of alkyl substituted Toc• radicals 1-9 in benzene (see Fig. 1). The rate, k_{-1}, was compared with k_1 for Reaction [1] and with k_3 for Reaction [3]. The effect of alkyl substituents on the aromatic ring of Toc• has also been studied. It is of interest to know how the rate of the reaction of ML-OOH with Toc• is different from that of the phospholipid hydroperoxide. We have thus measured the rate constant, k_{-1}, for the reaction of phosphatidylcholine hydroperoxide (PC-OOH) with a Toc• radical in benzene, *tert*- butanol and in micellar dispersions, and compared the observed k_{-1} values with the corresponding values for ML-OOH. The effect of the phytyl side chain on the reaction rate of Toc• in micellar dispersion also has been studied. In Figure 1 we give the structures of the molecules studied in this work. The study was intended to provide a basis for the interpretation of similar reactions in more complex biological systems.

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Abbreviations: LH, polyunsaturated fatty acid(s) and/or polyunsaturated fatty acid ester(s); LOOH, lipid hydroperoxide; LOO•, peroxyl radical; ML-OOH, methyl linoleate hydroperoxide; PC, egg phosphatidylcholine; PC-OOH, phosphatidylcholine hydroperoxide; PhO•, 2,6-di-*tert*-butyl-4-(4'-methoxyphenyl)phenoxyl; TLC, thin-layer chromatography; TocH, tocopherol; Toc•, tocopheroxyl radical.

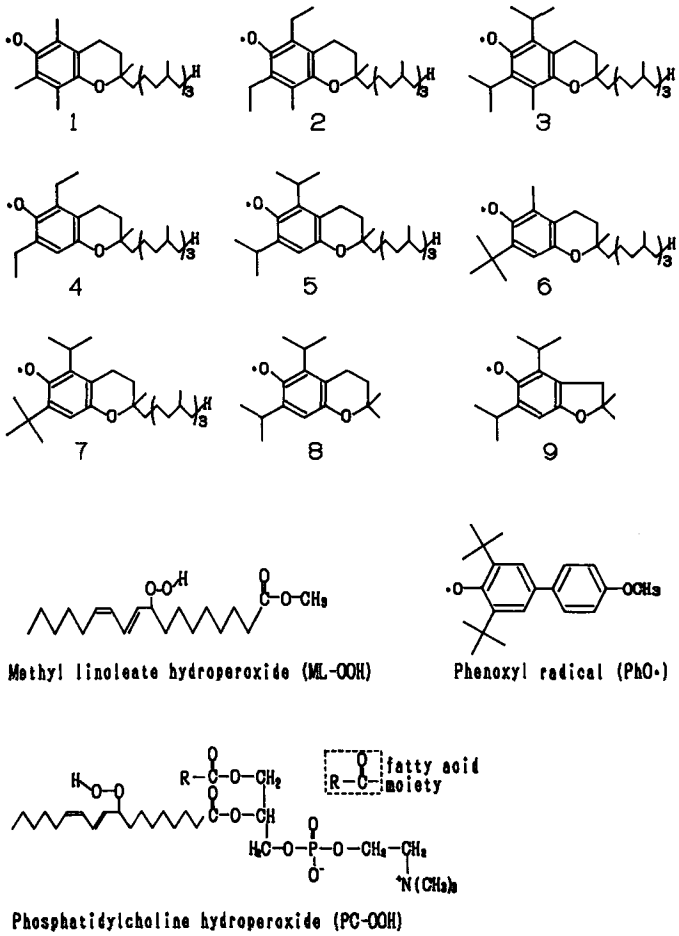

FIG. 1. Molecular structures of tocopheroxyl radicals 1–9, PhO•, ML-OOH (methyl linoleate hydroperoxide) and PC-OOH (phosphatidylcholine hydroperoxide).

MATERIALS AND METHODS

Sample preparation. Methyl linoleate (linoleic acid methyl ester, LH) (>99%) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Hydroperoxide levels in methyl linoleate were estimated by measuring absorption at 234 nm (15) and were found to be less than 0.3%. Egg phosphatidylcholine (PC) (>95%) was kindly supplied by Nippon Oil Fats Co. (Amagasaki, Japan). The average molecular weight of the PC was assumed to be 774 (13). The amount of hydroperoxides in the sample was found to be negligible as judged by thin-layer chromatography (TLC). α-TocH was supplied by Eisai Co. (Tokyo, Japan). Triton X-100 was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and was used as received. The 2,6-di-*tert*-butyl-4-(4′-methoxyphenyl)phenoxyl (PhO•) **10** was prepared by the method of Rieker and Scheffler (16).

ML-OOH was prepared by photosensitized oxidation of methyl linoleate using methyleneblue as a sensitizer (17). The oxidation product was purified by SiO_2 column chromatography. PC-OOH was prepared by the oxidation of PC with $^1O_2$, following the method of Terao *et al.* (18). The hydroperoxide was separated from the oxidation products by reverse-phase liquid chromatography. The average molecular weight of the PC-OOH was assumed to be 806, based on that of PC (774).

The preparation of TocH **2p–9p** was reported previously (19–21). The Toc• radicals **3, 5, 6, 7, 8** and **9** are fairly stable, and were prepared by PbO_2 oxidation of the corresponding TocH in benzene solution under a nitrogen atmosphere. However, in the cases of TocH **1p, 2p** and **4p**, the Toc• radicals **1, 2** and **4** produced were not very stable, and absorption spectra decreased rapidly with time. Therefore, Toc• **1, 2** and **4** were prepared by reaction between the stable PhO• radical **10** and the corresponding TocH in benzene at 25°C under a nitrogen atmosphere and were reacted immediately with ML-OOH solution.

Micellar dispersions of Triton X-100 (5.0 wt%) containing Toc• **5** were prepared as follows (22). 5,7-Diisopropyltocopherol **5p** (15–20 mg, 32–42 μmol) was dissolved in 5-mL of diethyl ether, and the solution was poured into a small flask. The diethyl ether was removed on a rotary evaporator to obtain a thin film on the flask wall. Twenty mL of aqueous Triton X-100 (5.0 wt%, in 0.1 M phosphate buffer, pH 7) was added, and the flask was shaken vigorously in a Vortex mixer for 1 min. PhO•-containing solutions of Triton X-100 (5.0 wt%) were prepared similarly and reacted with the above TocH-containing micellar dispersions. The PhO• radical is very stable in the absence of 5,7-diisopropyltocopherol and shows absorption peaks at $\lambda_{max} = 377$ and 577 nm in aqueous Triton X-100 dispersions (5.0 wt%). Upon mixing the micellar dispersion of TocH (0.66 mM) with the micellar dispersion of the PhO• radical (0.60 mM) (1:1, vol/vol) at 25.0°C, the absorption spectrum characteristic of the PhO• radical immediately changed to that of Toc• **5**. The new absorption maxima in the visible region ($\lambda_{max} = 397$ and 417 nm) are due to Toc• **5** (23). Because Toc• **5** is stable at 25.0°C, the absorption intensity decreases only gradually with time.

Measurements. The kinetic data were obtained on a Shimadzu UV-2100S (Kyoto, Japan) spectrophotometer by mixing equal volumes of solutions of Toc• and ML-OOH (or PC-OOH). The oxidation reactions were studied under pseudo-first-order conditions, and the observed rate constants (k_{obsd}) were calculated in the usual way, using standard least-squares analysis. All measurements were performed at 25.0°C, because Toc• **1, 2** and **4** used are not stable at 37°C.

RESULTS AND DISCUSSION

Reaction between ML-OOH and Toc• 1–9 in benzene. 5,7-Diisopropyltocopheroxyl **5** is comparatively stable in the absence of ML-OOH and shows absorption peaks at $\lambda_{max} = 417$ nm and 397 nm in benzene (Fig. 2). When adding a benzene solution of excess ML-OOH to a benzene solution of Toc•, the absorption spectrum of the Toc• gradually disappeared. Figure 2 shows an example of the results of the interaction between 5,7-diisopropyltocopheroxyl **5** (*ca.* 0.15 mM) and ML-OOH (21.5 mM) in benzene. The rate was measured by following the decrease in absorbance at 417 nm of Toc• **5**. Under these conditions, the rate of disappearance of Toc• in the presence of a constant concentration of ML-OOH is first-order in regard to Toc•. The pseudo-first-order rate constants, k_{obsd}, were obtained by varying the concentration of ML-OOH:

$$-d[Toc^•]/dt = k_{obsd}[Toc^•] \quad [5]$$

Toc• **5** shows a slow natural decay in benzene. Therefore,

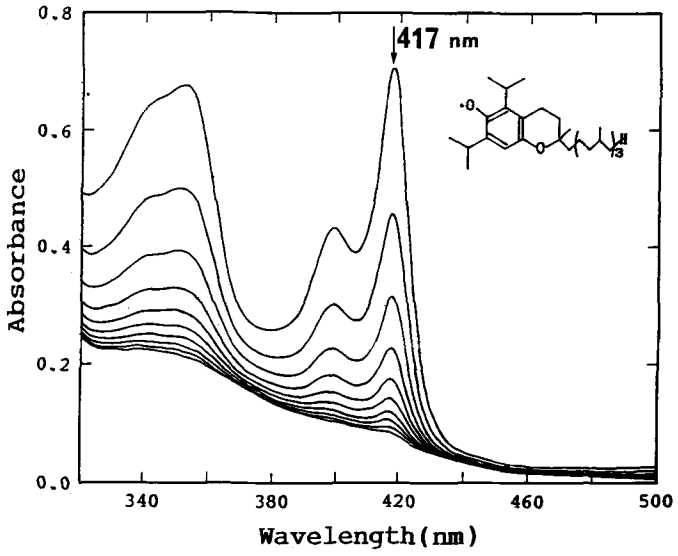

FIG. 2. Changes in the absorption spectrum of 5,7-diisopropyltocopheroxyl radical 5 in the course of the reaction of Toc• 5 with ML-OOH in benzene at 25.0°C. $[Toc^{\bullet}]_{t=0}$ = *ca.* 0.15 mM and $[ML\text{-}OOH]_{t=0}$ = 21.5 mM. The spectra were recorded at 100-s intervals. Arrow indicates decrease (↓) of absorbance with time. Abbreviation as in Figure 1. TOC•, tocopheroxyl radical.

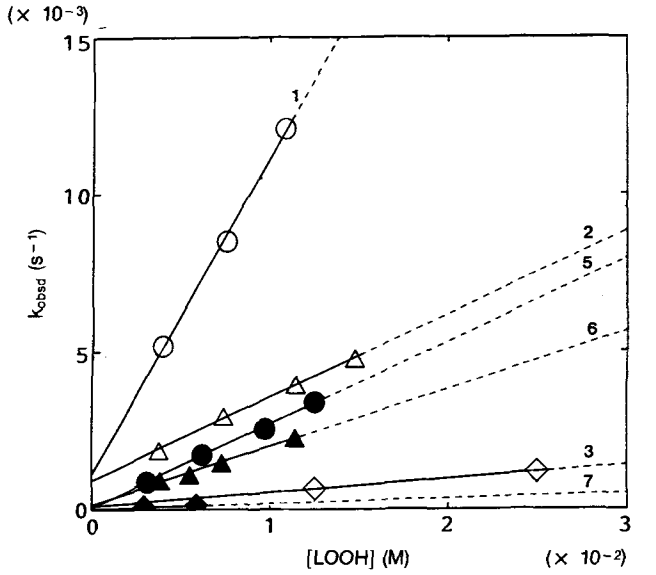

FIG. 3. Dependence of the pseudo-first-order rate constants, k_{obsd}, of Toc• radicals 1, 2, 3, 5, 6 and 7 on the concentration of ML-OOH in benzene at 25.0°C. Abbreviations as in Figures 1 and 2.

the pseudo-first-order rate constant, k_{obsd}, for Toc• bleaching is given by Equation 6:

$$k_{obsd} = k_o + k_s\,[LOOH] \qquad [6]$$

where k_o is the rate constant for the natural decay of Toc• in benzene solution, and k_s is the apparent second-order rate constant for the reaction of Toc• with added ML-OOH. These rate parameters are obtained by plotting k_{obsd} against [ML-OOH]. Similar measurements were made for the reactions of Toc• 1-9 with ML-OOH. As shown in Figure 3, the first-order decay constant, k_{obsd}, is proportional to the concentration of ML-OOH.

As reported by Mahoney and DaRooge (24,25), the results are consistent with Reactions [7] and [2]:

$$Toc^{\bullet} + LOOH \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} TocH + LOO^{\bullet} \qquad [7]$$

$$LOO^{\bullet} + Toc^{\bullet} \xrightarrow{k_2} LOO\text{-}Toc \qquad [2]$$

where $k_2[Toc^{\bullet}] >> k_1[TocH]$; k_2 is the second-order rate constant for the reaction of Toc• with LOO•. A combination product of Toc• and LOO•, that is, LOO-Toc, was isolated by Yamauchi *et al.* (26). Under these conditions, the rate of disappearance of Toc• is given by the expression (24,25):

$$-d[Toc^{\bullet}]/dt = \{k_o + 2k_{-1}[LOOH]\}\,[Toc^{\bullet}] \qquad [8]$$

The values of k_{-1} calculated from k_{obsd} are listed in Table 1. The experimental errors in the k_{-1} value for Toc• radicals 3, 5, 6, 7, 8 and 9 were less than ±7%. The natural decays of Toc• 1, 2 and 4 are much faster, and therefore we could only estimate the approximate rate constants.

Several investigators have measured the rate constant, k_1, for the reaction between LOO• and vitamin E derivatives (Reaction [1]) (27,28, and references therein). The most reliable k_1 value was reported by Burton *et al.* (27), using the inhibited autoxidation of styrene method. For example, the k_1 value obtained for the reaction of α-TocH with poly(peroxystyryl)peroxyl radical was $3.20 \times 10^6 M^{-1}s^{-1}$ in chlorobenzene at 30°C. Therefore, the k_{-1} value ($5.0 \times 10^{-1} M^{-1}s^{-1}$) obtained for α-Toc• 1 in the present study is about seven orders of magnitude lower than the above-mentioned k_1 value.

Effect of alkyl substituents in Toc• radicals. In the present work, we have determined the second-order rate constants, k_{-1}, for the reaction of ML-OOH with Toc• 1-9 in benzene. As listed in Table 1 for the Toc• radicals 4, 5, 6 and 7, having two alkyl substituents at the *ortho* positions to the OH group, the k_{-1} values obtained in benzene are $2.5 \times 10^{-1} M^{-1}s^{-1}$ for radical 4, $1.33 \times$

TABLE 1

Second-Order (k_{-1} and k_3) Rate Constants for the Reaction of Toc• 1-9 with Methyl Linoleate Hydroperoxide and Methyl Linoleate, Respectively, in Benzene at 25.0°C and Relative Rate Constants (k_{-1}/k_3)

Toc•	k_{-1}[a] ($M^{-1}s^{-1}$)	k_3[a] ($M^{-1}s^{-1}$)	k_{-1}/k_3
1	5.0×10^{-1}	—	—
2	1.3×10^{-1}	2.3×10^{-2}	5.7
3	2.11×10^{-2}	3.27×10^{-3}	6.45
4	2.5×10^{-1}	5.0×10^{-2}[b]	5.0
5	1.33×10^{-1}	1.86×10^{-2}[b]	7.15
6	9.14×10^{-2}	1.61×10^{-2}[b]	5.68
7	7.77×10^{-3}	1.10×10^{-3}[b]	7.06
8	1.31×10^{-1}	1.83×10^{-2}	7.16
9	8.41×10^{-2}	2.01×10^{-2}	4.18

[a]Experimental errors in k_{-1} and k_3 values were less than 7% for Toc• 3 and 5-9 and 20% for Toc• 1, 2 and 4. Toc•, tocopheroxyl radical.

[b]These values were previously reported (Ref. 13).

$10^{-1}M^{-1}s^{-1}$ for **5**, $9.14 \times 10^{-2}M^{-1}s^{-1}$ for **6** and $7.77 \times 10^{-3}M^{-1}s^{-1}$ for **7**. The values of k_{-1} decrease in the order of **4** > **5** > **6** > **7**, as the size of the two *ortho*-alkyl groups in the Toc• radical increases. The Toc• **4** is 32-times as reactive as the Toc• **7**. The results clearly indicate that the decrease in k_{-1} is due to steric factors. Inductive effects on the reaction rate of these Toc• radicals with ML-OOH would not be significant, because methyl, ethyl, isopropyl and *tert*-butyl groups have electron-donating properties that are not too different.

Further, the rate constants of Toc• radicals **1**, **2** and **3**, which have two alkyl substituents at the *ortho* positions and a methyl substituent at C-8 decrease in the order **1** > **2** > **3**. This result also suggests that the effect of steric hindrance on the reaction rate is considerable.

Both 5,7-diisopropyl-8-methyltocopheroxyl **3** and 5,7-diisopropyltocopheroxyl **5** have two isopropyl substituents at the ortho positions and, in addition, **3** has a methyl substituent at the *meta* position. When comparing the rate constant, k_{-1}, observed for Toc• **5** with that for Toc• **3**, compound **5** is 6.3 times more reactive than **3**. Similarly, 5,7-diethyltocopheroxyl **4** is 1.9 times as reactive as the 5,7-diethyl-8-methyltocopheroxyl **2**. The results indicate that substitution on the aromatic ring by an electron-donating methyl group (at C-8) results in a decrease in the second-order rate constant, k_{-1}. In other words, in the reaction between the Toc• and ML-OOH, the Toc• acts as an electron acceptor, and thus the rate constants will decrease as the total electron-donating capacity of the alkyl substituents on the aromatic ring of the Toc• increases (29).

Effect of alkyl side chain of Toc• radical on its reaction rate in benzene and in micellar dispersion. In order to clarify the effect of the phytyl side chain of the Toc• radical on the reaction rate, we have measured the rate constants, k_{-1}, for the reaction of ML-OOH with 5,7-diisopropyltocopheroxyl **5** and 5,7-diisopropyltocopheroxyl model **8** in benzene and in micellar dispersion.

As shown in Table 2, the Toc• **5** and Toc• **8** without phytyl side chain in 2-position reacted with the ML-OOH at a similar rate in benzene. On the other hand, in 5 wt% Triton X-100 micellar dispersion, the k_{-1} value ($6.93 \times 10^{-2}M^{-1}s^{-1}$) of Toc• **5** is 0.61 times that ($1.14 \times 10^{-1}M^{-1}s^{-1}$) of Toc• **8**. The result shows that the phytyl side chain of vitamin E has little effect on reactivity in homogeneous solution, whereas it has considerable effect on reactivity in micellar dispersion (1,2,30–33). The significance of the above changes in the rate constants, k_{-1}, is not clear at present, but it may relate to the biological activity of TocH. For instance, although both α-TocH models and α-TocH have high *in vitro* antioxidant activity in solution, the former has no *in vivo* vitamin E activity (34,35).

Reaction between PC-OOH and Toc• 5. It is of interest to examine whether or not the rate constant of hydrogen abstraction of PC-OOH is the same as that of ML-OOH, which is one of the hydroperoxide derivatives (LOOH) of the fatty acid moieties contained in the PC.

The rate constants for hydrogen abstraction from PC-OOH and ML-OOH by Toc• **5** (k_{obsd}) have been measured in benzene, *tert*-butanol and 5 wt% Triton X-100. The second-order rate constants, k_{-1}, are obtained by plotting k_{obsd} *vs.* [LOOH]. The k_{-1} values obtained are given in Table 3.

The observed k_{-1} value of PC-OOH was only 8% of that of ML-OOH in the nonpolar benzene solvent, whereas the k_{-1} value of PC-OOH was 1.9 times larger than that of ML-OOH in the more polar *tert*-butanol solvent. Furthermore, in micellar dispersion, PC-OOH reacted at a rate that was only about 30% of that observed for ML-OOH. There is, as yet, no unambiguous explanation for this apparent contradiction, but some possible explanations are offered in the following.

Firstly, the formation of reverse micelles in benzene solution must be considered, as PC molecules are known to aggregate into reverse micelles in nonpolar solvents (36,37). The solubility of Toc• **5**, which has a nonpolar phytyl side-chain, is high in benzene, but low in PC liposome. Thus, formation of reverse micelles might prevent Toc• **5** from reacting with the hydroperoxide group of PC-OOH, causing a smaller rate constant, k_{-1}, for PC-OOH than for ML-OOH. This would be in agreement with the results obtained for the hydrogen abstraction reaction from PC and from unsaturated fatty acids (LH) by Toc• **5** in benzene (13). Therefore, we measured the rate constant, k_{-1}, in polar *tert*-butanol solvent in which PC-OOH does not form reverse micelles. As the two fatty acid moieties are quite close in PC-OOH, Toc• **5** may not

TABLE 2

Pseudo-First-Order (k_{obsd}) and Second-Order (k_{-1}) Rate Constants for the Reaction of Toc• 5 and 8 with ML-OOH in Benzene or 5 wt% Triton X-100 (pH = 7.0, 0.1 M phosphate buffer) at 25.0°C

	Toc• 5			Toc• 8		
Solution	[ML-OOH] (mM)	k_{obsd} (s^{-1})	k_{-1}[a] ($M^{-1}s^{-1}$)	[ML-OOH] (mM)	k_{obsd} (s^{-1})	k_{-1}[a] ($M^{-1}s^{-1}$)
Benzene	3.12	8.81×10^{-4}	1.33×10^{-1}	4.68	1.42×10^{-3}	1.31×10^{-1}
	6.24	17.3		9.37	2.79	
	9.37	25.5		17.64	4.86	
	12.49	33.9				
Micellar dispersion	3.96	5.29×10^{-4}	6.93×10^{-2}	4.02	9.6×10^{-4}	1.14×10^{-1}
	7.46	9.82		7.37	16.9	
	11.22	15.3		11.08	24.5	
				14.69	34.3	

[a]For each Toc•, the experimental error in k_{-1} value was less than 7%. Toc•, tocopheroxyl radical; ML-OOH, methyl linoleate hydroperoxide.

TABLE 3

Pseudo-First-Order (k_{obsd}) and Second-Order (k_{-1}) Rate Constants for the Reaction of Toc• 5 with ML-OOH and PC-OOH in Benzene, *tert*-butanol or 5 wt% Triton X-100 (pH = 7.0, 0.1 M phosphate buffer) at 25.0°C

	ML-OOH			PC—OOH		
Solution	[ML-OOH] (mM)	k_{obsd} (s^{-1})	k_{-1}[a] ($M^{-1}s^{-1}$)	[PC-OOH] (mM)	k_{obsd} (s^{-1})	k_{-1}[a] ($M^{-1}s^{-1}$)
Benzene	3.12	8.81×10^{-4}	1.33×10^{-1}	2.68	9.61×10^{-5}	1.09×10^{-2}
	6.24	17.3		5.09	14.4	
	9.37	25.5		7.66	20.9	
	12.49	33.9		9.97	25.2	
tert-Butanol	3.92	8.56×10^{-5}	2.49×10^{-3}	2.92	6.37×10^{-5}	4.68×10^{-3}
	7.47	10.5		5.52	7.91	
	11.51	12.5		7.71	10.1	
	15.00	14.1		10.35	13.3	
Micellar dispersion	3.96	5.29×10^{-4}	6.93×10^{-2}	3.05	2.60×10^{-4}	2.02×10^{-2}
	7.46	9.82		5.99	3.65	
	11.22	15.3		7.98	4.61	

[a]Experimental error in k_{-1} values was less than 7%. Abbreviations as in Figure 1.

be able to easily approach the active site, that is, the -OOH group of PC-OOH. In such a case, the k_{-1} value of PC-OOH will be smaller than that of ML-OOH. However, the observed k_{-1} value ($4.68 \times 10^{-3}M^{-1}s^{-1}$) of PC-OOH was 1.9 times larger than that ($2.49 \times 10^{-3}M^{-1}s^{-1}$) of ML-OOH in *tert*-butanol. The reason for this is not clear at present.

In Triton X-100, PC-OOH reacted with Toc• 5 at a rate that was only about 30% of that observed with ML-OOH. As the accessibility of the ML-OOH appears to be greater than that of PC-OOH in micellar dispersion, the reactivity of ML-OOH was higher than that of PC-OOH.

The cause of the prooxidant effect of TocH. We recently reported the second-order rate constants, k_3, for the reactions of the Toc• radicals 4–7 with methyl linoleate (LH) in benzene using a spectrophotometric monitoring system (Reaction [3]) (12,13). We have also measured the rate constants, k_3, for the reactions of Toc• 1, 2, 3, 8 and 9 with LH in benzene at 25.0°C. As described in a previous section, α-Toc• 1 is unstable, and we could not determine the rate constant, k_3. The rate constants, k_3, obtained are summarized in Table 1, together with those reported for Toc• 4, 5, 6 and 7. The results indicate that the effect of substitution on the reaction rates, k_3, observed for Toc• 2–9 is very similar to that on k_{-1}. The values of k_{-1} were plotted against k_3. As shown in Figure 4, the k_{-1} values were found to correlate linearly with the k_3 values (correlation coefficient = 0.96). The ratio of k_{-1} to k_3 was estimated to be about 6 ± 1 from the values given in Table 1 (Eq. 9).

$$k_{-1} = (6 \pm 1) \times k_3 \quad [9]$$

It is well known that tocopherols (vitamin E) are present in biomembranes and in oils, where they function as antioxidants. The antioxidant properties of TocH have been ascribed to the initial oxidation by the LOO• radical of the phenolic hydroxyl group, producing a Toc• radical (Reaction [1]) (1,2). On the other hand, several investigators have demonstrated that α-TocH at high concentrations acts as a prooxidant during the autoxidation of LH in aqueous medium and in bulk phase (3–11). This prooxidant effect of α-TocH leads to an increase of the level of LOOH with a conjugated diene structure. Loury *et al.* (3) and Terao and Matsushita (9) have proposed that Toc• radicals participate in this prooxidant effect through Reactions [3] and [4].

In the present work, we have measured the rate constants, k_{-1}, for the reaction of ML-OOH with Toc• 1–9. The k_{-1} values were found to be about six times larger than those (k_3) of the corresponding Toc• radicals for Reaction [3]. The results suggest that both Reactions [3] and [4] may relate to the prooxidant effect of α-TocH at high concentrations. Therefore, if LH coexists with LOOH in edible oils or in membranes, the rate of disappearance of Toc• will be represented by Equation 10:

$$-d[Toc•]/dt = k_3[LH][Toc•] + k_{-1}[LOOH][Toc•] \quad [10]$$

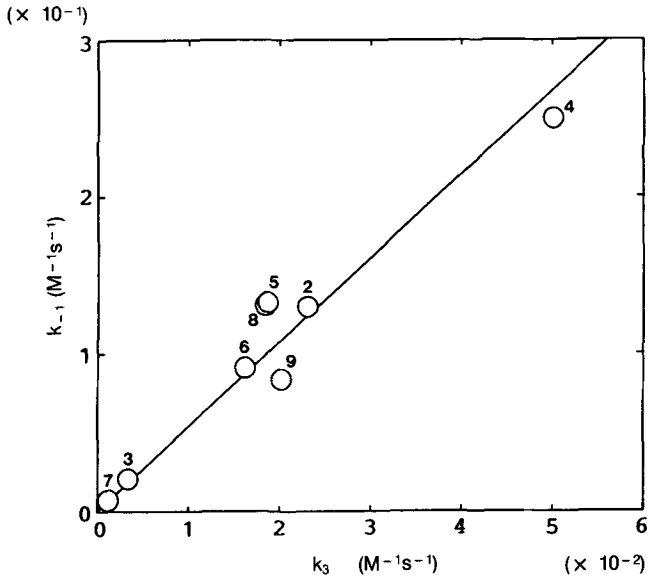

FIG. 4. Plot of k_{-1} *vs.* k_3 for tocopheroxyl radicals 2–9.

In the initial stage of lipid degradation, the concentration of LOOH will be much lower than that of LH, and thus the second term in Equation 10 is negligible. Consequently, the prooxidant effect of α-TocH in edible oils and fats will be induced by the hydrogen abstraction Reaction [3] between Toc• and LH. On the other hand, if the autoxidation proceeds, the level of LOOH increases. When the concentration of LOOH approaches approximately 17% of that of LH, the radical decay Reactions [3] and [4] will approach similar rates. These facts suggest that not only the chain transfer Reaction [3] is due to Toc•, but also that Reaction [4] between Toc• and LOOH participates in the prooxidant effect of α-TocH.

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Kinetic Study of Reactions Between Tocopheroxyl Radicals and Fatty Acids

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A kinetic study on the prooxidant effect of vitamin E derivatives has been carried out. Rates of hydrogen abstraction from various fatty acids and egg yolk lecithin by tocopheroxyl radicals were determined spectrophotometrically. The rate constants measured in micellar dispersion were compared with those obtained in homogeneous solutions. The effects of structural variations of the vitamin E derivatives on their prooxidant activities were examined. The formation of lecithin reverse micelles in benzene appears to prevent the tocopheroxyl radicals from reacting with the phospholipid fatty acid moieties.
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It is well known that vitamin E (α-, β-, γ- and δ-tocopherols) inhibits the autoxidation of organic molecules in liquid phase. The mechanism involved has been studied extensively by several investigators (1–4). Vitamin E is present in cellular membranes and in edible oils and acts as an antioxidant by protecting polyunsaturated fatty acids and other lipids from peroxidation.

The antioxidant properties of tocopherols (TocH) have been ascribed to hydrogen transfer from the OH group in the TocH to a peroxyl radical (LOO•). The hydrogen transfer produces a tocopheroxyl radical (Toc•) which combines with another LOO• (reactions [1] and [2]) (5,6).

$$\text{LOO}\bullet + \text{TocH} \xrightarrow{k_1} \text{LOOH} + \text{Toc}\bullet \quad [1]$$

$$\text{LOO}\bullet + \text{Toc}\bullet \rightarrow \text{Nonradical products} \quad [2]$$

On the other hand, several investigators have shown that α-TocH in high concentration acts as a prooxidant during the autoxidation of polyunsaturated fatty acids (LH) (7,8). This prooxidant effect of α-TocH leads to an increase of hydroperoxides with a conjugated diene structure. Loury *et al.* (7) and Terao and Matsushita (8) proposed that Toc• radicals participate in this prooxidant effect through the following reactions [3] and [4]:

$$\text{Toc}\bullet + \text{LH} \xrightarrow{k_3} \text{TocH} + \text{L}\bullet \quad [3]$$

$$\text{Toc}\bullet + \text{LOOH} \xrightarrow{k_{-1}} \text{TocH} + \text{LOO}\bullet \quad [4]$$

where reaction [3] is a hydrogen abstraction from LH and a chain transfer reaction. Reaction [4] is a reversal of reaction [1]. These reactions are important in the understanding of the antioxidant and prooxidant properties of vitamin E. However, so far the kinetics of these reactions have not been studied extensively.

We have recently determined the rate constant k_{-1} for reactions of alkyl hydroperoxides with Toc• (9,10). We also determined the second-order rate constant k_3 for reactions of various fatty acid esters with Toc• (11,12). The rate k_3 was compared with k_{-1}. In order to interpret observed features of k_3 quantitatively, we carried out *ab initio* calculations of models for the fatty acid esters. Calculated results were consistent with the experimental results. It was shown that the observed features of k_3 can be explained in terms of the pseudo-π-conjugation between the C=C double bond and the active hydrogen-carbon bond. The effect of substituent groups at the 5- and 7-positions of Toc• was also studied. Furthermore, we measured k_3 of egg yolk lecithin.

There remain, however, a few questions that still need to be answered. Firstly, it would be worthwhile to know how k_3 values change when going from homogeneous solutions to inhomogeneous systems, such as micellar dispersions. Although a direct *in vivo* approach to the study of the prooxidant reaction is very difficult, it should prove useful to study such inhomogeneous lipid systems, because the information that can be obtained may be relevant to membranes. It would be of interest, for example, to measure k_3 of egg yolk lecithin in micelles. Secondly, the effect of structural variation of TocH on prooxidant activity has not been studied previously. Information about the effects of the phytyl side chain, the heterocyclic ring and the methyl at the 8-position will be needed.

We have carried out a kinetic study of the prooxidant effect of TocH. Rates of hydrogen abstraction from various fatty acids and egg yolk lecithin by Toc• were determined spectrophotometrically.

MATERIALS AND METHODS

Stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4n-6), *cis*-4,7,10,13,16,19-docosahexaenoic acid (22:6n-3), their methyl esters (Med18:0, etc.), and their ethyl esters (Et18:0, etc.) (>99% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). In order to estimate the ratios of hydroperoxides in the unsaturated fatty acids and their esters, we measured the absorption at 234 nm due to the hydroperoxides (12). Hydroperoxide levels in the samples were found to be lower than 0.3%. In the present work, we assumed that the k_3 values of the fatty acids are close to those of the corresponding esters. In fact, since the carboxyl group is located far from the active site (allylic hydrogen) in the fatty acids, esterification may not have a large influence on k_3.

As reported previously (9), vitamin E radicals are not stable, and thus stable radicals were used for the present work. The preparation of 5,7-diisopropyl-tocopherol (di-iPr-TocH), 6-hydroxy-2,2-dimethyl-5,7-diisopropyl-chromane

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Abbreviations: Et, ethyl; Et18:0, stearic acid ethyl ester; Et18:1, oleic acid ethyl ester; Et18:2, linoleic acid ethyl ester; Et18:3, linolenic acid ethyl ester; Et20:4n-6, arachidonic acid ethyl ester; Et22:6n-3, *cis*-4,7,10,13,16,19-docosahexaenoic acid ethyl ester; iPr, isopropyl; LH, lipid; LOO•, lipid peroxyl; Me, methyl; Me18:0, stearic acid methyl ester; Me18:1, oleic acid methyl ester; Me18:2, linoleic acid methyl ester; Me18:3, linolenic acid methyl ester; Me20:4n-6, arachidonic acid methyl ester; Me22:6n-3, *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester; tBu, *t*-butyl; TocH, tocopherol and its analogues; TocHM, 6-hydroxy-2,2-dimethyl-chromane and its analogues; TocH5, 2,3-dihydro-5-hydroxy-2,2-dimethyl-benzofuran and its analogues; Toc•, tocopheroxyl radical and its analogues.

(di-iPr-TbcHM) and 2,3-dihydro-5-hydroxy-2,2-dimethyl-4,6-diisopropyl-benzofuran (di-iPr-TbcH5) has been described previously (13–15). 5,7-Diisopropyl-8-methyl-tocopherol (di-iPr-8-Me-TbcH) was prepared according to published methods (16). 6-Hydroxy-2,2-dimethyl-5,7-diisopropyl-8-methyl-chromane (di-iPr-8-Me-TbcHM) was synthesized by reaction of paraformaldehyde with di-iPr-TbcHM in 1,4-dioxane (16,17). 5,7-Diethyl-8-methyl-tocopherol (di-Et-8-Me-TbcH) and *d*-α-TbcH were kindly supplied by Dr. Shiro Urano of Tokyo Metropolitan Institute of Gerontology and Eisai Co, Ltd., respectively, both from Tokyo, Japan. In the experiments done in homogeneous solution, the radicals (di-iPr-Tbc• etc) were prepared by PbO_2 oxidation of the corresponding TbcH in a nitrogen atmosphere. Tbc• radical containing micellar dispersions of Triton X-100 were prepared as reported (18).

Egg yolk lecithin was isolated according to the method reported by Singleton *et al.* (19). Lecithin fatty acid moieties were analyzed by gas chromatography of the methyl esters obtained by transmethylation of lecithin. The values obtained are as follows: palmitic acid (16:0), 41.3%; palmitoleic acid (16:1), 2.5%; 18:0, 8.7%; 18:1, 29.5%; 18:2, 16.5%; and 20:4n-6, 1.7%. The average molecular weight of the lecithin was estimated to be 763.7. The amount of hydroperoxides in the sample was found to be negligible as judged by thin-layer chromatography.

Commercial Triton X-100 was used without further purification. The aqueous solution (1.0 wt%) was buffered at pH 7.0 by use of KH_2PO_4 and $NaHPO_4$. Benzene and *t*-butanol were distilled before use.

The experimental procedures used for measurement of the rate constants were described previously in detail (9–12). Briefly, the kinetic data were obtained with a Shimadzu UV-2100S spectrophotometer by mixing equal volumes of solutions of 18:0, etc., or egg yolk lecithin and Tbc• under a nitrogen atmosphere. The molar concentration of Tbc• and LH before the beginning of reaction was 5×10^{-5} and $1\text{–}3 \times 10^{-3}$ M, respectively. All measurements were performed at 25.0 ± 0.5°C; Tbc• is unstable at 37°C.

The pseudo-first-order rate constant for reaction [3] (k_{obsd}) was determined by following a decrease in absorbance of Tbc• around 420 nm; k_{obsd} is given by equation [5]:

$$k_{obsd} = k_0 + k_3 [LH] \qquad [5]$$

where k_0 denotes the rate constant for natural decay of Tbc•, k_3 stands for the second-order rate constant for reactions of 18:0, Me18:0, Et18:0, etc., or egg yolk lecithin (LH) with Tbc• and [LH] refers to the molar concentration of LH. The rate parameters were obtained by plotting k_{obsd} against [LH]. Experimental errors were less than ±5%.

RESULTS AND DISCUSSION

The k_3 values of the reactions of 18:0, Me18:0, etc., with di-iPr-Tbc• in micellar dispersion and *t*-butanol are given in Table 1. As reported previously (11,12), k_3 increases as the number of C=C double bonds in the fatty acids and fatty acid esters increases (18:0<18:1<18:2<18:3<20:4n-6< 22:6n-3 and Me18:0<Me18:1<18:2<18:3<20:4n-6< Me22:6n-3). Allylic hydrogen abstraction plays a major role in the high reactivity of the unsaturated fatty acid and the ester in reaction [3]. The allylic hydrogen is activated by the π-electron system (C=C double bond).

The rate k_3 of 18:1 (Me18:1) is much smaller than that of 18:2 (Me18:2). While 18:1 (Me18:1) has four hydrogen atoms activated by a single π-electron system, 18:2 (Me18:2) has two hydrogen atoms activated by two π-electron systems. Thus, the two hydrogen atoms activated by two π-electron systems will contribute to the high reactivity of 18:2 (Me18:2). As reported previously (12), the k_3 observed can be explained in terms of the pseudo-π-conjugation between the C=C double bond and the active hydrogen-carbon bond.

The free acids 18:2, 18:3, 20:4n-6 and 22:6n-3 (or Me18:2, Me18:3, Me20:4n-6 and Me22:6n-3) have two, four, six and ten hydrogen atoms activated by two π-electron systems, respectively. The rate constants per active hydrogen (k_{abstr}/H's) are given in Table 1. In *t*-butanol, k_{abstr}/H values for Me18:2, Me18:3, Me20:4n-6 and Me22:6n-3 are similar. However, in micellar dispersion, k_{abstr}/H decreases as the number of C=C double bond in 18:2, 18:3, 20:4n-6 and 22:6n-3 increases. This could be explained as follows: The micelles contain Tbc• and LH which have a polar "head" (-O• and -COOR, respectively) and a long hydrocarbon "tail". The polar "head" is located on the outer surface of the micelle facing the water phase. Since the polar "head" of Tbc• (-O•) cannot easily approach the hydrophobic end of the long hydrocarbon "tail" of LH in the micelles, the probability of a reaction occuring with an

TABLE 1

The k_3 Values and k_{abstr}/H for the Reaction of Fatty Acid Derivatives with di-iPr-Toc•[a]

	Benzene[b]		*t*-Butanol[c]		Micelles[d]	
	k_3 $M^{-1}s^{-1}$	k_{abstr}/H $M^{-1}s^{-1}$	k_3 $M^{-1}s^{-1}$	k_{abstr}/H $M^{-1}s^{-1}$	k_3 $M^{-1}s^{-1}$	k_{abstr}/H $M^{-1}s^{-1}$
18:0	$< 10^{-5}$	—	—	—	—	—
18:1	1.04×10^{-5}	2.60×10^{-6}	$\approx 10^{-5}$	—	5.4×10^{-2}	1.3×10^{-2}
18:2	1.82×10^{-2}	9.10×10^{-3}	1.01×10^{-2}	5.07×10^{-3}	2.4×10^{-1}	1.2×10^{-1}
18:3	3.84×10^{-2}	9.60×10^{-3}	2.40×10^{-2}	6.00×10^{-3}	3.6×10^{-1}	8.9×10^{-2}
20:4n-6	4.83×10^{-2}	8.05×10^{-3}	3.72×10^{-2}	6.20×10^{-3}	4.4×10^{-1}	7.4×10^{-2}
22:6n-3	9.05×10^{-2}	9.05×10^{-3}	6.52×10^{-2}	6.50×10^{-3}	7.4×10^{-1}	7.4×10^{-2}

[a]iPr, isoproply; Toc•, tocopheroxylradical.
[b]Reference 12, obtained for Et18:0, etc.
[c]Obtained for Me18:0, etc.
[d]Obtained for 18:0, etc.

active hydrogen located near the tip of the "tail" is small. Accordingly, k_{abstr}/H is not the same for 18:2, 18:3, 20:4n-6 and 22:6n-3 in micellar dispersions.

The idea that the Toc• radicals and lipid chains within micelles are locally restricted may, of course, be somewhat simplistic, as micelles are dynamic structures. Also diffusion control between micelles might affect k_3 values here, especially for Tocs• with phytyl side chains.

When LH and Toc• are contained in micelles, the actual local concentration of LH and Toc• are much larger than the nominal concentration of the solution. As a result, the apparent second-order rate constant k_3 ($M^{-1}s^{-1}$) in micellar dispersions is larger than that in homogeneous solutions. Since the specific gravity of Triton X-100 is close to that of water, and the concentration is 1.0% by weight, k_3 in micellar dispersion must become larger than that in *t*-butanol by about two orders of magnitude. However, as shown in Table 1, k_3 in micellar dispersion is larger than that in *t*-butanol by only one order of magnitude. This supports our view that the active site of Toc• (-O•) cannot easily access that of LH (allylic hydrogen) which is concealed behind Triton X-100, which prevents Toc• from reacting with LH.

The k_3 values of the reactions of Toc• with Me18:2 are given in Table 2 together with the rate constants for the antioxidant reaction of TocH with a phenoxyl, k_s, (14,15, 20–22). Di-iPr-Toc• and di-iPr-8-Me-Toc• have prooxidant reactivity similar to that of di-iPr-TocM• and di-iPr-8-Me-TocM•, respectively, in benzene. As in the case of the antioxidant reaction (20), the effect of the phytyl side chain on the prooxidant activity is not very pronounced in homogeneous solution.

In the antioxidant reaction, k_s increases when going from TocH with a 6-membered heterocyclic ring to the corresponding TocH with a 5-membered ring (di-iPr-TocM•< di-iPr-Toc5•). This can be explained in terms of an increase in orbital overlap between the 2p type lone pair on the ring oxygen and the aromatic π electrons (21). However, in the prooxidant reaction, di-iPr-TocM• having a 6-membered heterocyclic ring has a reactivity similar to that of di-iPr-Toc5• having a 5-membered ring. The effect of the heterocyclic ring on the prooxidant activity is not significant.

Table 2 shows that the bulkier the substituent groups at the 5- and 7-positions of Toc•, the smaller k_3 [5-isopropyl-7-*t*-butyl-tocopheroxyl radical (iPr-tBu-Toc•) <di-iPr-Toc•<5,7-diethyl-tocopheroxyl radical (di-Et-Toc•) and di-iPr-8-Me-Toc•<di-Et-8-Me-Toc•<*d*-*α*-Toc•]. As reported previously (12), the pronounced substituent effect in the prooxidant reaction in homogeneous solution is due to the steric hindrance by the substituent groups at the 5- and 7-positions. In contrast, the steric hindrance does not have a large influence on the antioxidant activity of TocH (14,15,20–22).

Methylation at 8-position of Toc• decreases k_3 (di-iPr-Toc•>di-iPr-8-Me-Toc•, di-iPr-TocM•>di-iPr-8-Me-TocM•, and di-Et-Toc•>di-Et-8-Me-Toc•), while methylation at 8-position of TocH increases k_s (di-iPr-TocH<di-iPr-8-Me-TocH, di-iPr-TocHM<di-iPr-8-Me-TocHM and di-Et-TocH<di-Et-8-Me-TocH). Its methylation increases and decreases the antioxidant and prooxidant activities of TocH, respectively. In this context, it is suggestive that all natural TocH have a methyl group at 8-position. The increase in k_s can be explained in terms of an increase in electron donating capacity of TocH and a charge transfer ability of the antioxidant reaction (21). At present, we cannot explain the decrease in k_3 upon methylation, but the following possibilities exist. Firstly, methylation at the 8-position may stabilize Toc• and reduce prooxidant activity. Then since k_3 of di-iPr-8-Me-Toc• (di-iPr-8-Me-TocM•) is much smaller than that of di-iPr-Toc• (di-iPr-TocM•), methylation may increase steric hindrance by the substituent groups at the 7-position. Figures 1a and b show a schematic sketch of molecular structures of di-iPr-Toc• and di-iPr-8-Me-Toc•, respectively. By methylation at the 8-position, the active site of Toc• (-O•) is concealed behind two methyl groups of the isopropyl group at the 7-position, which could prevent Toc• from reacting with LH.

The effect of the phytyl side chain of Toc• on the prooxidant activity was examined also in micellar dispersion. In benzene, the k_3 values of di-iPr-Toc• and di-iPr-TocM• are similar. However, in micellar dispersion, k_3 of di-iPr-Toc• is much smaller than k_3 of di-iPr-TocM•. We believe that this can be explained in terms of the structure of the micelles. Since the polar "head" of di-iPr-Toc• (-O•) can probably not gain easy access to the tip of the long hydrocarbon "tail" of LH in the micelle, the

TABLE 2

The k_3 Values for the Reactions of Tocopheroxyl Radical (Toc•) with 18:2 and k_s Values for Tocopherol and its Analogues.

	k_3		
	Benzene[a] $M^{-1}s^{-1}$	Micelles[b] $M^{-1}s^{-1}$	k_s $M^{-1}s^{-1}$
di-iPr-Toc•	1.86×10^{-2}[c]	2.4×10^{-1}	2.51×10^{3}[d]
di-iPr-TocM•	1.83×10^{-2}	9.5×10^{-1}	2.82×10^{3}[e]
di-iPr-8-Me-Toc•	3.27×10^{-3}	—	4.43×10^{3}[f]
di-iPr-8-Me-TocM•	3.24×10^{-3}	—	3.01×10^{3}[g]
di-iPr-Toc5•	2.01×10^{-2}	—	5.40×10^{3}[h]
di-Et-Toc•	4.97×10^{-2}[c]	—	1.97×10^{3}[d]
di-Et-8-Me-Toc•	3.69×10^{-2}	—	3.64×10^{3}[f]
d-*α*-Toc•	$\approx 5 \times 10^{-2}$[i]	—	5.12×10^{3}[j]
Me-tBu-Toc•[k]	1.61×10^{-2}[c]	—	2.97×10^{3}[d]
iPr-tBu-Toc•	1.10×10^{-3}[c]	—	2.39×10^{3}[g]

[a]Obtained for Me18:2.
[b]Obtained for 18:2.
[c]Reference 12.
[d]Reference 14.
[e]Reference 20.
[f]Reference 21.
[g]Unpublished data.
[h]Reference 15.
[i]Since *d*-*α*-Toc• is not stable, experimental error is very large.
[j]Reference 22.
[k]5-Methyl-7-*t*-butyl-tocopherol.

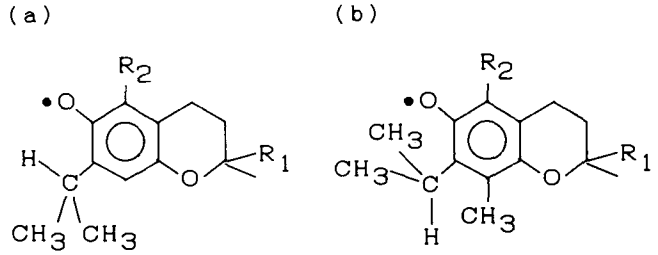

FIG. 1. Molecular structures of di-iPr-Toc• (a) and di-iPr-8-Me-Toc• (b). Abbreviations as in Table 1.

TABLE 3

The k_{leci} Values obtained for the Reaction with di-iPr-Toc• and k_{expd} Data

	k_{leci} $M^{-1}s^{-1}$	k_{expd} $M^{-1}s^{-1}$
Benzene[a]	7.02×10^{-3}	2.07×10^{-2}
t-Butanol	4.95×10^{-3}	4.56×10^{-3}
Micelle	1.0×10^{-1}	1.3×10^{-1}

[a]Reference 12. Abbreviations as in Table 1.

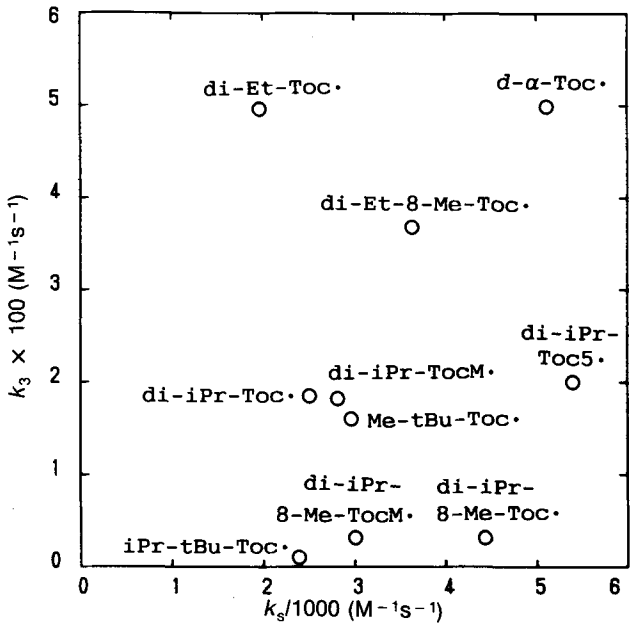

FIG. 2. Plot of k_3 obtained in benzene *vs.* k_s.

probability of the reaction of the active hydrogen located near the tip of the "tail" is small. However, this is not the case for di-iPr-TocM• which does not have a phytyl side chain and thus is more mobile in the micelles. Accordingly, k_3 of di-iPr-TocM• is larger than that of di-iPr-Toc•. The effects of the phytyl side chain on the mobility in heterogeneous system, such as micelle and membranes, have already been the subject of some experimental studies (1,23,24).

It is interesting to examine whether or not the second-order rate constant of the hydrogen abstraction of egg yolk lecithin (k_{leci}) is the same as that expected for the fatty acid moieties of lecithin (k_{expd}). The k_{leci} values obtained in the reaction with di-iPr-Toc• in benzene, *t*-butanol and in micelles are given in Table 3 together with the k_{expd} data. The k_{expd} was calculated as described previously (12). The k_{expd} values in benzene, *t*-butanol and micellar dispersion were estimated by the use of k_3 of Et18:0, etc. (12), Me18:0, etc. and 18:0, etc. (Table 1), respectively. In *t*-butanol and micelles, k_{leci} is similar to the corresponding k_{expd}. Steric hindrance between the two fatty acid moieties of lecithin is unlikely to affect the kinetics. However, in benzene, k_{leci} is about 30% of the corresponding k_{expd}. Lecithins are prone to aggregate as reverse micelles in nonprotic solvents such as benzene (25,26), but not in *t*-butanol (25). Formation of reverse micelles prevents di-iPr-Toc• from reacting with the fatty acid moiety in benzene.

It is desirable to compare k_s and k_3 of various TocH in order to find useful antioxidants. Figure 2 shows a plot of k_3 obtained in benzene *vs.* k_s. A useful antioxidant should satisfy the requirement that k_s is large and k_3 is small. From Figure 2, it is suggested that di-iPr-Toc5• meets these criteria. TocH with bulky substituents at 5- and 7-positions and TocH with a methyl group at 8-position should also be useful.

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The Urinary Excretion of Thiobarbituric Acid Reactive Substances and Malondialdehyde by Normal Adult Males After Consuming a Diet Containing Salmon

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In this study we investigated the output of thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA), as thiobarbituric acid (TBA)–MDA adduct, in the urine from subjects eating a diet in which the only source of n-3 long-chain, polyunsaturated fatty acids was fresh salmon. Nine healthy men, ages 30–65, were confined in the United States Department of Agriculture Western Human Nutrition Research Center, San Francisco, CA, for 100 d; food intake and exercise levels were controlled. All subjects were placed on a stabilization diet (StD) for 20 d, then six were fed the salmon diet for 40 d. The others remained on the StD. The groups switched diets for the last 40 d. Both diets were isocaloric (16% protein, 54% CHO and 30% fat by energy %). The salmon diet contained 7.5% of calories from n-6 fatty acids (FAs) and 2% from n-3 FAs, primarily eicosapentaenoic acid and docosahexaenoic acid in a 50:60 ratio, while the StD contained 7.5% from n-6 FAs and < 0.3% n-3 FAs (with presumably no significant amounts of C_{20} or C_{22} n-3 FAs). Twenty-four hour urinary output was collected, and 2% 3-d pool samples prepared for analysis of urinary TBARS and the TBA–MDA adduct. The total urinary output of each individual varied considerably, and on a daily basis the concentration of autoxidation products in an individual's urine varied also. However, the mean daily output (in μmoles TBA–MDA equivalents/day) at the end of the salmon diet feeding period was significantly greater (7.05 ± 1.33 TBARS, $P < 0.05$; and 7.07 ± 1.73 TBA–MDA adduct, $P < 0.01$) compared to when the subjects were eating the StD (5.65 ± 1.09 TBARS and 4.65 ± 0.76 TBA–MDA adduct). When the TBARS and TBA–MDA adduct values were normalized relative to creatinine output (in nmoles TBA–MDA equivalents/μmole creatinine), the data achieved even greater statistical significance. The mean output of the group eating the salmon diet was 0.478 ± 0.076 for TBARS ($P < 0.01$) and 0.476 ± 0.082 for the TBA–MDA adduct ($P < 0.001$) *vs.* 0.345 ± 0.059 for TBARS and 0.283 ± 0.041 for the TBA–MDA adduct when the subjects were consuming the StD. Thus, the consumption of cooked fish may increase one's exposure to MDA and other autoxidation products, compounds that may be carcinogenic or mutagenic.
Lipids 28, 757–761 (1993).

Fish oil supplements and diets containing fish have been purported to promote cardiovascular health and alleviate several other human afflictions, such as cancer, arthritis and psoriasis (1–5). Some investigators have questioned the adequacy of the antioxidant level in the typical Western diet if individuals start consuming increased amounts of n-3 long-chain polyunsaturated fatty acids (FAs) (6–9). An experimental approach to answer this question was attempted by Draper and colleagues (10) who measured the malondialdehyde (MDA) levels in urine from normal volunteers who had consumed fish oil supplements. They found that MDA excretion in the urine was increased after consumption of a fish oil supplement but concluded that this increase was the result of autoxidation products in the supplement rather than an increased production of these substances *in vivo*. They suggested that fish oil supplements that were not previously autoxidized do not necessarily increase the amount of MDA produced *in vivo*, although they did state that protracted consumption of n-3 FAs must be presumed to increase the vitamin E requirement. There is no comparable information about the excretion of lipid autoxidation products in the urine after consumption of fresh fish.

In a recent study (11,12) directed primarily toward the effect of a diet with increased n-3 polyunsaturated FAs on FA metabolism, blood lipids and platelet function, 24-h urine samples were collected and stored at −70°C (no urine was lost). The source of the n-3 long-chain polyunsaturated FAs in this study was fresh salmon rather than a fish oil supplement. The stored urine samples provided an opportunity to examine the excretion of thiobarbituric acid reactive substances (TBARS) and MDA in urine samples from human volunteers eating a diet containing fresh fish. The results of these analyses are reported here.

MATERIALS AND METHODS

Materials. All foods were purchased at local food markets. The salmon (Pacific, chinook, *Oncorhynchus tshawytscha*) was purchased fresh from a seafood wholesale supply company as a large batch from a single boat. It was frozen until used for meal preparations during the study.

Subjects. Twelve volunteers, consisting of men between the ages of 30 and 65, were recruited from the West Coast. All volunteers were given complete physical examinations. Body weights had to be within −10 to +20% of ideal body weights using the Metropolitan Life Insurance Company tables (medium frame values from the 1983 edition). Evidence of existing illness or chronic disease was an exclusion criterion. Smoking, excessive alcohol consumption or evidence of narcotic abuse were also exclusionary. The physical characteristics of the final nine volunteers who completed the study were previously published (11).

Experimental design. All the volunteers were confined to the Nutrition Research Unit of the Western Human Nutrition Research Center, San Francisco, CA during the study. A crossover design was used. The subjects were

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Abbreviations: En%, energy percent; FA, fatty acid; HPLC high-performance liquid chromatography; MDA, malondialdehyde; RDA, Recommended Dietary Allowance; StD, stabilization diet; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TMP, 1,1,3,3-tetramethoxy-propane.

fed a stabilization diet (StD), containing less than 1% n-3 long-chain, polyunsaturated FAs for twenty days. On day 21 of the study, the subjects were divided into two groups. One, designated Group A, was then placed on the salmon diet for 40 d. The other group, designated Group B, remained on the StD for forty more days. On day 61, the groups switched diets for the remaining 40 d of the study. Statistical comparisons of the measured parameters were made using day 20 as the baseline value for each subject in group A and day 60 for each subject in group B. Subjects served as their own controls.

Diets. The diets consisted of foodstuffs accessible in grocery stores in San Francisco, CA. No dietary supplements were given except α-tocopherol to bring the diet to 200% of the Recommended Dietary Allowance (RDA) for this vitamin because diets containing large amounts of polyunsaturated FA may deplete the reserves of antioxidant nutrients. (A complete description of the diets, listing all the major and minor nutrients, is available upon request.) There was a five-day menu cycle used throughout the study. No alcohol was included in the diets. The diets were designed around a prudent diet concept of 30:55:15 fat, carbohydrate and protein in energy percent (En%). The nutrient composition of the diet was calculated from a computerized nutrient data bank and adjusted to provide at least the RDA for known essential nutrients. Dietary quantities of saturated and n-6 FAs were held constant in these diets. The StD had less than 1% n-3 FAs (mostly as α-linolenic acid) and approximately 50% monounsaturated FAs (mostly oleic acid). The salmon diet had approximately 7.5% long-chain polyunsaturated n-3 FAs with a concomitant reduction in the level of monounsaturated FAs. All participants were weighed every day, and the caloric value of their meals adjusted daily, if necessary, to maintain their weight within the design limits of the study (±2%). If all the intakes for each volunteer were averaged during the entire study, the mean energy intake was approximately 2800 Kcal/d. As no two subjects ate the same amount of salmon each day, the amount of n-3 FAs is best expressed in terms of percent of calories per day rather than grams of n-3 FAs consumed per day. In this study each volunteer fed the salmon diet received 2.1% of his calories from long-chain, n-3 polyunsaturated FAs. If, for example, an individual in this study was receiving 3000 Kcal/d, he would have consumed about 2.1 g of 20:5n-3, 0.8 g of 22:5n-3 and 3.0 g of 22:6n-3, or about 6 g/d of n-3 polyunsaturated FAs.

Analysis of urinary TBARS, thiobarbituric acid (TBA)—MDA adduct and creatinine. Urine samples were stored frozen at −70°C until thawed for analysis. Fresh aliquots were taken from the 3-d pools for each analysis. TBARS were determined by the method of Buege and Aust (13). Aliquots of urine were added to the TBA reagent (0.375% TBA, 15% trichloroacetic acid, 0.25N HCl and 0.2% butylated hydroxytoluene). Samples and standards were heated in a water bath at 80°C for 30 min, cooled in ice water and centrifuged for 10 min at 1000 × *g*. Absorbance of the supernatant was read at 535 nm, and the concentration of TBA-MDA equivalents was calculated from a standard curve derived from the acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP).

Since the TBARS assay for human tissues and body fluids is subject to interference by substances other than MDA (14), a TBA test based on high-performance liquid chromatography (HPLC) was also used for comparison. The TBA-MDA adduct (assumed to Form in a 2:1 ratio, see Ref. 14) was determined by a modification of the method of Tatum *et al.* (15). Aliquots of urine were added to TBA reagent (0.27% TBA 0.13N HCl and 0.2% butylated hydroxytoluene). Samples and standards were heated at 80°C for 30 min, cooled, and centrifuged for 10 min at 1000 × *g*. The TBA-MDA complex was extracted in butanol, mixed with 1/2 vol of methanol and injected into the HPLC. HPLC conditions were as follows: mobile phase, 1:1 (vol/vol) mixture of methanol and 0.05% tetrabutyl ammonium dihydrogen phosphate as the ion-pairing reagent; column 5 μ C_{18} reversed phase (Beckman Instruments, Fullerton, CA); flow rate, 1 mL/min and detection, fluorimeter set at an excitation wavelength of 550 nm. The TBA-MDA adduct was calculated from a standard of TMP carried through the same procedure.

Creatinine in the urine was measured by an alkaline picrate colorimetric procedure in a Centrifichem System 600 (Baker Instruments, Allentown, PA) using reagents purchased from Trace America, Inc. (Miami, FL).

Statistical analysis. The data were analyzed with a two-tailed, paired *t*-test. All statistical analyes were done with the PC SAS data system, Version 6.03 (SAS Institute, Chapel Hill, NC 1988).

RESULTS

Tables 1 and 2 give the results of the TBARS test and the TBA-MDA adduct analyses (in μmoles TBA-MDA equivalents/day), respectively, for the nine subjects who completed this study. The total urinary output of each individual varied considerably, consequently the daily concentration of autoxidation products in the urine also varied. Despite this variation, the mean daily output of oxidation products at the end of the salmon diet feeding period was significantly greater (approximately 20% for the TBARS and 50% for TBA-MDA adduct) than the output of these substances when the subjects were eating the StD. The TBARS test combined means were significantly different at the $P < 0.05$ level while the TBA-MDA adduct means were significantly different at the $P < 0.01$ level.

When the TBARS test and the TBA-MDA adduct values were normalized relative to the creatinine content of the urine (nmoles TBA-MDA equivalents/μmole creatinine), the data achieved greater statistical significance. This is shown in Tables 3 and 4 for the TBARS test and the TBA-MDA adduct, respectively. Combined mean urinary output for all subjects on the salmon diet was 50% greater for the TBARS test ($P < 0.01$) and 75% percent greater for the TBA-MDA adduct analysis ($P < 0.001$) than the combined means before the subjects consumed the salmon diet for forty days.

The above results were obtained using the combined data from Groups A (n = 6) and B (n = 3) to give a total n of nine for the study. As this was a crossover study, Group A returned to the stabilization diet for the last 40 d of the study. It is apparent from the tables that both the TBARS and TBA-MDA adduct values for Group A in the urine at day 100 of the study had returned to their pre-salmon feeding values (day 20). For Group A the day 100 values for the TBARS test (Table 1) were lower than

TABLE 1

Analysis of TBARS in Urine of Subjects Consuming Salmon[a]

Subject	Group	Baseline (day 20 Group A and day 60 Group B)	End of intervention (day 60 Group A and day 100 Group B)	End of study (day 100)
		(μmoles TBA-MDA equivalents/day)		
1	A	4.27	7.86	4.62
2	A	6.21	7.54	3.31
3	A	7.15	6.40	4.26
4	A	6.85	8.87	6.80
11	A	4.97	6.28	4.78
12	A	6.75	7.32	4.78
Means ± SD, Group A		6.03 ± 1.16	7.38 ± 0.96	4.76 ± 1.14
5	B	4.78	7.99	—
8	B	4.82	4.23	—
10	B	5.26	6.98	—
Means ± SD, Group B		4.95 ± 0.27	6.40 ± 1.95	—
Combined means ± SD		5.65 ± 1.09	7.05 ± 1.33	—

[a]Differences between the combined means, using baseline day 20 and end day 60 for Group A and baseline day 60 and end day 100 for Group B, were significant at the $P < 0.05$ level. Abbreviations: TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; MDA, malondialdehyde.

the 20 day values, but this difference vanished when the TBARS values were normalized relative to daily creatinine output (Table 3).

DISCUSSION

It has been established that increased MDA ingestion from oxidized fish oil increases the excretion of MDA in the urine (10,16,17). The work presented here shows that the ingestion of cooked fish containing n-3 long-chain polyunsaturated FAs increases the excretion of both TBARS and MDA in the urine. Both methods used to measure MDA in the urine (the colorimetric TBARS test and the fluorimetric HPLC determination of the TBA-MDA adduct) gave essentially the same results when the subjects were consuming the salmon diet, but the TBARS test yielded somewhat higher values than the TBA-MDA adduct determination while the subjects were fed the StD diet. This suggests the presence of interfering substances in the urine when the subjects were fed the StD diet. Under these conditions, therefore, measurement of urinary MDA by HPLC of the TBA-MDA adduct is more accurate than the TBARS test. Kosugi *et al.* (18) reported that HPLC separation was necessary for accurate quantitation of the red pigment formed in the TBA assay of human urine.

The quantity of MDA excreted daily in the urine by our North American male subjects (eight Caucasians and one Asian) agrees closely with the amounts found recently in the urine of Japanese subjects when expressed on a body

TABLE 2

Analysis of TBA-MDA Adduct in Urine of Subjects Consuming Salmon[a]

Subject	Group	Baseline (day 20 Group A and day 60 Group B)	End of intervention (day 60 Group A and day 100 Group B)	End of study (day 100)
		(μmoles TBA-MDA equivalents/day)		
1	A	4.40	9.96	4.72
2	A	4.02	7.32	4.35
3	A	5.31	6.73	4.09
4	A	5.53	7.86	6.60
11	A	4.12	6.11	3.81
12	A	6.00	6.98	3.27
Means ± SD, Group A		4.90 ± 0.83	7.49 ± 1.34	4.76 ± 1.14
5	B	4.00	8.45	—
8	B	4.32	3.65	—
10	B	4.13	6.58	—
Means ± SD, Group B		4.15 ± 0.16	6.23 ± 2.42	—
Combined means ± SD		4.65 ± 0.76	7.07 ± 1.73	—

[a]Differences between the combined means, using baseline day 20 and end day 60 for Group A and baseline day 60 and end day 100 for Group B, were significant at the $P < 0.01$ level. See Table 1 for abbreviations.

TABLE 3

Analysis of TBARS in Urine of Subjects Consuming Salmon[a]

Subject	Group	Baseline (day 20 Group A and day 60 Group B)	End of intervention (day 60 Group A and day 100 Group B)	End of study (day 100)
		(nmoles TBA-MDA equivalents/μmol creatinine)		
1	A	0.285	0.414	0.260
2	A	0.458	0.534	0.264
3	A	0.344	0.471	0.323
4	A	0.290	0.490	0.267
11	A	0.337	0.477	0.314
12	A	0.415	0.600	0.447
Means ± SD, Group A		0.354 ± 0.069	0.498 ± 0.063	0.312 ± 0.071
5	B	0.368	0.478	—
8	B	0.310	0.329	—
10	B	0.301	0.512	—
Means ± SD, Group B		0.326 ± 0.036	0.439 ± 0.097	—
Combined means ± SD		0.345 ± 0.059	0.478 ± 0.076	—

[a]Differences between the combined means, using baseline day 20 and end day 60 for Group A and baseline day 60 and end day 100 for Group B, were significant at the $P < 0.01$ level. See Table 1 for abbreviations.

weight basis. Our subjects (mean of 84 ± 13 kg, see Ref. 11) excreted 55 and 84 nmoles of TBA-MDA equivalents/kg/day when fed the stabilization and salmon diets, respectively (HPLC analysis of TBA-MDA adduct). Twelve healthy Japanese subjects (six males and six females with an overall mean body weight of 53 kg) excreted from 26 to 95 nmoles of red pigment/kg/day (TBA-reactive material in urine separated by HPLC, see Ref. 18).

MDA is a mutagen in the Ames test (19) and is thought to be a carcinogen (20). However, because ingested MDA usually becomes conjugated to lysine during the absorption process (16), it may not be as hazardous as MDA produced *in vivo* from n-3 long-chain polyunsaturated FAs. Thus, the question arises whether the MDA found in the urine of these subjects was produced *in vivo* or in the cooking process before the fish was eaten. Siu and Draper (21) have suggested that less than 10% of the ingested MDA is excreted in the urine. Unfortunately, no measurements of the MDA content of the cooked fish were made in this study so that the endogenous or exogenous origin of the TBARS and MDA excreted in the urine cannot be established with any certainty. However, we believe that it is unlikely that the MDA was present in the fresh fish as it was kept frozen at −20°C until it was ready for cooking. (Commercial salmon fishing boats usually keep the fish alive in salt water or packed in ice until they dock. The fish in this study were filleted and frozen at a dockside processing plant within hours of docking.) Also, the fish fillets were not flame exposed during cooking to minimize oxidation. Fish, because of its high water content and low fat content, cooks internally at 100°C or less,

TABLE 4

Analysis of TBA-MDA Adduct in Urine of Subjects Consuming Salmon[a]

Subject	Group	Baseline (day 20 Group A and day 60 Group B)	End of intervention (day 60 Group A and day 100 Group B)	End of study (day 100)
		(nmoles TBA-MDA equivalents/μmol creatinine)		
1	A	0.294	0.524	0.266
2	A	0.297	0.518	0.348
3	A	0.256	0.495	0.310
4	A	0.234	0.435	0.259
11	A	0.279	0.465	0.250
12	A	0.369	0.572	0.395
Means ± SD, Group A		0.288 ± 0.046	0.501 ± 0.048	0.305 ± 0.058
5	B	0.308	0.506	—
8	B	0.278	0.284	—
10	B	0.236	0.483	—
Means ± SD, Group B		0.274 ± 0.036	0.424 ± 0.122	—
Combined means ± SD		0.283 ± 0.041	0.476 ± 0.082	—

[a]Differences between the combined means, using baseline day 20 and end day 60 for Group A and baseline day 60 and end day 100 for Group B, were significant at the $P < 0.001$ level. See Table 1 for abbreviations.

largely independent of the set cooking temperature. It should be noted also that the diet contained twice the RDA of tocopherol. Work by Draper and colleagues (8,10) and others (22,23) suggests that added tocopherol will suppress the oxidation of long-chain polyunsaturated FAs *in vivo*. Also, as it is known that the amount of free MDA in the diet, plasma and urine is negligible, the mutagenicity and carcinogenicity of dietary MDA may not be a major problem. The lysine adduct of MDA, the form in which it is absorbed, is nonmutagenic. This does not mean, however, that dietary n-3 FAs cannot be potential risk factors because they could lead to increased lipid peroxidation *in vivo*. The peroxides could then produce MDA in tissues where it could react with cellular DNA.

Kaasgaard *et al.* (24) reported that livers from Cynomolgus monkeys after feeding menhaden oil showed variable but increased lipofuscin levels despite a diet that had been supplemented with a fourfold excess of tocopherol. This suggests that excess tocopherol at that level may not halt lipid peroxidation *in vivo*.

The subjects in this study may have either ingested or produced much more MDA than was detected in the urine if the speculation of Siu and Draper (21) concerning the small percent of MDA excreted in urine is correct. Whether the increased excretion of TBARS and MDA found here indicates any adverse health consequence related to consumption of food containing n-3 polyunsaturated FAs remains to be determined. However, none of the subjects in this short term study showed any adverse effects ascribable to lipid oxidation. Their *in vitro* platelet aggregation as measured by sensitivity to adenosine diphosphate and arachidonic acid was reduced, and their *in vivo* bleeding times were unchanged. The total serum cholesterol was unchanged but their very low density lipoprotein and triglycerides levels were reduced and their high-density lipoprotein cholesterol level went up significantly. Their risk factor profile was in fact healthier at the end of the study by the usual criteria, such as lipid profiles, platelet function, etc. The complete report on the health parameters studied in the volunteers is found in References 11 and 12. If subsequent work does show that the increased urinary excretion of lipid peroxidation byproducts is the result of *in vivo* processes, increased intake of antioxidant nutrients, preferably through dietary food sources containing these compounds, may be warranted.

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COMMUNICATION

Novel Brominated Phospholipid Fatty Acids from the Caribbean Sponge *Agelas* sp.

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The very long-chain fatty acids, (5*E*,9*Z*)-6-bromo-5,9-tetracosadienoic, (5*E*,9*Z*)-6-bromo-23-methyl-5,9-tetracosadienoic, (5*E*,9*Z*)-6-bromo-5,9-pentacosadienoic and (5*E*,9*Z*)-6-bromo-24-methyl-5,9-pentacosadienoic acids, were identified in the phospholipids (mainly phosphatidylethanolamine) of the sponge *Agelas* sp. Structure elucidation was accomplished by means of mass spectrometry and chemical transformations, including deuteration with Wilkinson's catalyst. All of the sterols from the sponge had the Δ5,7 nucleus, with 24-methylcholesta-5,7,22-trien-3β-ol (ergosterol) and 24-ethylcholesta-5,7,22-trien-3β-ol being the most abundant.
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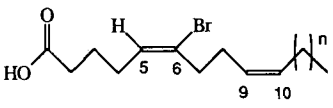
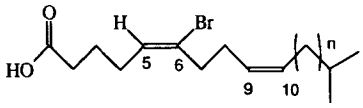
Phospholipids with brominated fatty acids are particularly rare in nature. Only two reports of naturally-occurring phospholipid brominated fatty acids have appeared and both were concerned with sponges. Two heptacosadienoic acids with the rare 5,9-diene pattern, *iso*/*anteiso* methyl branching, and the bromovinyl function were isolated from the marine sponges *Petrosia ficiformis* and *Petrosia hebes* (1). Likewise, the long-chain fatty acid (5*E*,9*Z*)-6-bromo-5,9-hexacosadienoic acid (**3a**) was also isolated from the phospholipids of a *Hymeniacidonid* sponge (2). It is significant to note that all of these fatty acids were found principally in phosphatidylethanolamine (PE) and that the bromine atom was always found at the vinylic carbon 6-position of the 5,9-diene functionality of the demospongic acid, regardless of carbon chain length or methyl branching.



1a n=13

2a n=14

3a n=15



2b n=12

3b n=13

SCHEME 1

These brominated phospholipid fatty acids in marine sponges are of particular interest, as the number of halogenated fatty acids found in marine organisms is extremely small in comparison with the number of halogenated secondary metabolites reported to date (2). A few other free halogenated long-chain fatty acids have been reported in the marine environment. Six fatty acid chlorohydrins of palmitic and stearic acids (mainly with the combinations 9-chloro-10-hydroxy and 10-chloro-9-hydroxy) were found in the total lipids of the jellyfish *A. aurita* (3). This finding was followed by the elegant work of Schmitz and Gopichand (4), who isolated (7*E*,13ζ,15*Z*)-14,16-dibromo-7,13,15-hexadecatrien-5-ynoic acid from the marine sponge *Xestospongia muta*, the first dibrominated straight-chain acetylenic acid from a sponge. Other examples include a monobrominated straight-chain C_{18} bisacetylenic acid from *X. testudinaria* (5) and six mono- and dibrominated straight-chain unsaturated C_9, C_{16} and C_{18} acids from the same genus (6). Although all of the latter reports are on free fatty acids, the only halogenated fatty acids in phospholipids have been found in sponges.

In our present work we wish to report the isolation and structural elucidation of four new brominated phospholipid fatty acids, namely (5*E*,9*Z*)-6-bromo-5,9-tetracosadienoic acid (**1a**), (5*E*,9*Z*)-6-bromo-5,9-pentacosadienoic acid (**2a**), (5*E*,9*Z*)-6-bromo-23-methyl-5,9-tetracosadienoic acid (**2b**) and (5*E*,9*Z*)-6-bromo-24-methyl-5,9-pentacosadienoic acid (**3b**), from the phospholipids of an *Agelas* sp., which was collected during a recent expedition to Mona Island, Puerto Rico. We also wish to report the unusual sterol composition of this sponge.

EXPERIMENTAL PROCEDURES

Agelas sp. was collected August 1, 1992, near Mona Island, Puerto Rico, at a depth of 25 m. The sponge was freeze-dried in a Labconco Freeze Dryer 4.5 (Model 77510; Kansas City, MO). The sponge (36 g) was carefully cleaned of all nonsponge debris and cut into small pieces. Extraction with 250 mL of chloroform/methanol (1:1, vol/vol) yielded the total lipids. The neutral lipids, glycolipids and phospholipids (20 mg) were separated by column chromatography on silica gel (60–200 mesh) using the procedure of Privett *et al.* (7). The phospholipid classes were fractionated by thin-layer chromatography (TLC) using silica gel 60 and chloroform/methanol/NH_4OH (65:35:5, by vol) as solvent. 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 column chromatographic purification and elution with hexane/diethyl ether (9:1, vol/vol). The resulting methyl esters were analyzed by gas chromatography/mass spectrometry (GC/MS) using a Hewlett-Packard 59970 MS ChemStation (Hewlett-Packard, Palo Alto, CA) equipped with a 30 m × 0.32 mm nonpolar fused silica column (Supelco, Bellefonte, PA) with SPB™-1 as the bonded phase. GC/Fourier transform infrared (FTIR) spectra were

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

recorded on a Nicolet (Madison, WI) 740 FTIR spectrometer. For the location of double bonds, pyrrolidides were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, vol/vol) in a capped vial (2 h at 100°C) followed by ethereal extraction from the acidified solution and purification by preparative TLC (9). The double bond position of the monoenoic acids was elucidated by preparing the corresponding dimethyl disulfide derivatives by dissolving the esters (2 mg) in dimethyldisulfide (0.2 mL) and adding a solution (0.05 mL) of iodine in diethyl ether (60 mg/mL) and heating the solution at 50°C for 24 h, followed by the standard work-up (10). Hydrogenations were carried out in 10 mL of absolute methanol in the presence of catalytic amounts of platinum oxide (PtO_2). Deuterations were also carried out in 10 mL of methanol in the presence of catalytic amounts of Wilkinson's catalyst [$RhCl(PPh_3)_3$]. Deuterium was obtained from the reaction of sodium and D_2O. Spectral data for the key fatty acids for this discussion follows.

Methyl (5*E*,9*Z*)-6-bromo-5,9-tetracosadienoate. MS (70 eV) *m/z* (relative intensity), 377(M^+-Br, 5.3), 236(4), 221(3), 204(3), 179(6.7), 177(2.9), 167(5.8), 161(10.7), 159(10.3), 149(14.7), 147(8.9), 141(9), 139(10), 135(14.3), 133(7.2), 131(7.3), 123(16), 121(11), 119)13), 111(16.8), 109(22.9), 107(12.8), 105(14), 97(37.6), 95(38), 91(27), 85(20), 83(47), 81(56), 79(43), 74(35), 71(46), 69(81), 67(42).

N-(5*E*,9*Z*)-6-bromotetracosa-5,9-dienoylpyrrolidine. MS (70 eV) *m/z* (relative intensity), 497($C_{28}H_{50}NOBr^+$, 0.1), 495(0.1), 416(M^+-Br, 18), 260(15.2), 258($C_{11}H_{17}NOBr^+$, 13.5), 180($C_{11}H_{18}NO^+$, 5.3), 126(9.5), 113(100), 98(25), 85(13.7).

Methyl (5*E*,9*Z*)-6-bromo-23-methyl-5,9-tetracosadienoate. MS (70 eV) *m/z* (relative intensity), 391(M^+-Br, 9.1), 359(M^+-Br-CH_3OH, 3.8), 250 (8.5), 221(3.3), 179(4.2), 177(3.2), 167(4.9), 161(13), 159(13), 149(17), 147(10), 141(18), 139(22), 135(16), 133(11), 131(5), 121(16), 119(16), 111(16), 109(24), 107(18), 105(13), 97(38), 95(35), 91(25), 85(17), 83(50), 81(70), 79(59), 74(33), 71(31), 69(66), 67(60).

N-(5*E*,9*Z*)-6-bromo-23-methyltetracosa-5,9-dienoylpyrrolidine. MS (70 eV) *m/z* (relative intensity), 511($C_{29}H_{52}$-$NOBr^+$, 0.18), 509(0.16), 430(M^+-Br, 24), 260(14.5), 258($C_{11}H_{17}NOBr^+$, 14.5), 180($C_{11}H_{18}NO^+$, 5), 126(7.2), 113(100), 98(25), 85(14).

Methyl (5*E*,9*Z*)-6-bromo-5,9-pentacosadienoate. MS (70 eV) *m/z* (relative intensity), 391(M^+-Br, 9), 359(M^+-Br-CH_3OH, 4.1), 250(7.3), 221(4.1), 179(4.1), 177(4.8), 167(4.2), 161(14), 159(14), 149(11), 147(13), 141(16), 139(17), 135(14), 133(8), 131(5), 121(12), 119(14), 111(12), 109(23), 107(19), 105(12), 97(30), 95(26), 91(18), 85(14), 83(38), 81(58), 79(55), 74(26), 71(26), 69(51), 67(51).

N-(5*E*,9*Z*)-6-bromopentacosa-5,9-dienoylpyrrolidine. MS (70 eV) *m/z* (relative intensity), 511($C_{29}H_{52}NOBr^+$, 0.38), 509(0.26), 430(M^+-Br, 42), 260(22), 258($C_{11}H_{17}NOBr^+$, 21), 180($C_{11}H_{18}NO^+$, 7), 126(7.6), 113(100), 98(32), 85(16).

Methyl (5*E*,9*Z*)-6-bromo-24-methyl-5,9-pentacosadienoate. MS (70 eV) *m/z* (relative intensity), 405(M^+-Br, 5.5), 373(M^+-Br-CH_3OH,1) 331(1.6), 264(4.4), 191(3.6), 179(3), 177(4), 167(2), 161(8.8), 159(8.6), 149(9.6), 147(7.5), 141(14), 139(11), 135(11), 133(5.7), 131(5.6), 121(12), 119(7.9), 111(21.6), 109(25), 107(9), 105(14), 97(47), 95(38), 91(14), 85(22), 83(55), 81(53), 79(44), 74(21), 71(45), 69(61), 67(46).

N-(5*E*,9*Z*)-6-bromo-24-methylpentacosa-5,9-dienoylpyrrolidine. MS (70 eV) *m/z* (relative intensity), 525($C_{30}H_{54}$-$NOBr^+$, 0.1), 523(0.1), 444(M^+-Br,17), 260(13), 258(C_{11}-$H_{17}NOBr^+$, 16), 180($C_{11}H_{18}NO^+$, 5.4), 126(6.7), 113(100), 98(20), 85(11.7).

RESULTS AND DISCUSSION

The principal phospholipid from *Agelas* sp. was identified by TLC as PE, but small amounts of phosphatidylserine, phoshatidylinositol and phosphatidylcholine were also detected. The major fatty acids from the phospholipids of the sponge were identified in the standard way from the GC equivalent chain length values (ECL) and electron impact mass spectra of the methyl esters and the *N*-acylpyrrolidides. The complete list of fatty acids identified is shown in Table 1. Branched-chain fatty acids (particularly

TABLE 1

Principal Phospholipid Fatty Acids from *Agelas* sp.[a]

ECL[b]	Fatty acid	Abundance wt%)
13.00	Tridecanoic (13:0)	6.2
13.61	12-Methyltridecanoic (*i*-14:0)	2.7
13.73	11-Methyltridecanoic (*ai*-14:0)	0.5
14.00	Tetradecanoic (14:0)	2.9
14.65	13-Methyltetradecanoic (*i*-15:0)	6.4
14.72	12-Methyltetradecanoic (*ai*-15:0)	3.6
15.00	Pentadecanoic (15:0)	1.3
15.45	Methylpentadecanoic (16:0)	0.7
15.63	14-Methylpentadecanoic (*i*-16:0)	1.6
15.73	9-Hexadecenoic (16:1)	1.7
16.00	Hexadecanoic (16:0)	6.5
16.35	15-Methyl-9-hexadecenoic (17:1)	5.9
16.45	Methylhexadecanoic (17:0)	5.8
16.64	15-Methylhexadecanoic (*i*-17:0)	2.8
16.71	14-Methylhexadecanoic (*ai*-17:0)	1.3
17.00	Heptadecanoic (17:0)	1.1
17.76	3,7,11,15-Tetramethylhexadecanoic (20:0)	9.6
17.90	11-Octadecenoic (18:1)	0.5
18.00	Octadecanoic (18:0)	3.6
18:43	Methyloctadecanoic (19:0)	5.2
18.62	17-Methyloctadecanoic (*i*-19:0)	0.5
18.83	11-Nonadecenoic (19:1)	2.4
19.00	Nonadecanoic (19:0)	0.6
19.32	5,8,11,14-Eicosatetraenoic (20:4)	3.3
20.00	Eicosanoic (20:0)	5.2
20.62	19-Methyleicosanoic (*i*-21:0)	1.4
20.71	18-Methyleicosanoic (*ai*-21:0)	2.1
21.00	Heneicosanoic (21:0)	2.8
21.64	20-Methylheneicosanoic (*i*-22:0)	0.3
22.00	Docosanoic (22:0)	0.6
22.61	21-Methyldocosanoic (*i*-23:0)	0.4
22.72	20-Methyldocosanoic (*ai*-23:0)	0.5
23.53	5,9-Tetracosadienoic (24:2)	0.6
24.16	23-Methyl-5,9-tetracosadienoic (*i*-25:2)	1.8
24.51	5,9-Pentacosadienoic (25:2)	0.6
25.50	5,9-Hexacosadienoic (26:2)	0.6
25.90	6-Bromo-5,9-tetracosadienoic (24:2)[c]	0.5
26.53	6-Bromo-23-methyl-5,9-tetracosadienoic (*i*-25:2)[c]	1.8
26.92	6-Bromo-5,9-pentacosadienoic (25:2)[c]	1.4
27.20	2-Hydroxyhexacosanoic (*h*-26:0)	0.8
27.52	6-Bromo-24-methyl-5,9-pentacosadienoic (*i*-26:2)[c]	0.4
27.90	6-Bromo-5,9-hexacosadienoic (26:2)	0.3

[a]The aldehyde heneicosanal (21:0) was also identified in the mixture (1.2%).

[b]Equivalent chain length (ECL) values are those of the methyl esters of the acids.

[c]These acids have not been described before in nature.

iso/*anteiso*) were predominant in the mixture, accounting for up to 56% of the total fatty acid composition. Of interest are the large amounts in this particular sponge of tridecanoic acid (13:0), which is not a common fatty acid in most sponges. Phytanic acid, namely 3,7,11,15-tetramethylhexadecanoic acid (20:0), was also observed in the mixture as the most abundant fatty acid (10%); no other isoprenoid fatty acid was detected in the sponge. The main demospongic fatty acids in the sponge, *i.e.*, those with the Δ5,9 diene function, were characterized as 5,9-tetracosadienoic acid, 5,9-pentacosadienoic acid and 5,9-hexacosadienoic acid. It is interesting to note that the recently discovered 23-methyl-5,9-tetracosadienoic acid was also identified in the mixture (2% relative abundance). The complete characterization of all of these fatty acids has been previously described (11).

The most interesting fatty acids in the sponge were a series of five brominated Δ5,9 demospongic acids. One of the fatty acids was identified as the 6-bromo-5,9-hexacosadienoic acid (**3a**), as confirmed by comparing its spectral data with that published in the literature (2). The other four brominated fatty acids in the phospholipids of *Agelas* sp. were further characterized as the corresponding 6-bromo derivatives of the principal demospongic acids present in the sponge. All of the brominated fatty acids showed similar spectral characteristics. The main feature of the ^1H nuclear magnetic resonance (NMR) spectrum was the presence of multiplets between 5.32 and 5.41 ppm for the C-9 and C-10 hydrogens, while the C-5 hydrogen was observed further downfield as a triplet at 5.84 ppm, due to the electronegative vicinal bromine at C-6. The GC/MS of the brominated fatty acid methyl esters showed strong mass spectral peaks at *m/z* 74 (due to the McLafferty rearrangement typical of fatty acid methyl esters) and M^+-79 peaks as molecular ions, indicating facile loss of bromine under electron impact.

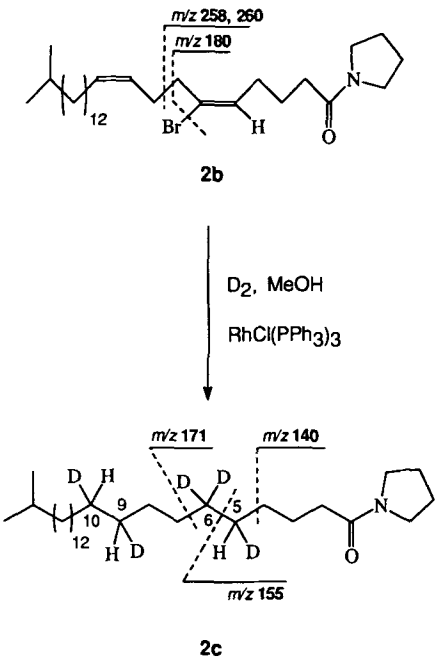

FIG. 1. Key mass spectral fragmentations for the N-(5*E*,9*Z*)-6-bromo-23-methyltetracosa-5,9-dienoylpyrrolidine (2b) and the N-[5,6,6,9,10-$^2H_5$]23-methyltetracosanoylpyrrolidine (2c).

The GC/MS of the corresponding brominated fatty acid pyrrolidides was critical for identifying the position of the bromine substituent. For example, N-(5*E*,9*Z*)-6-bromo-23-methyltetracosa-5,9-dienoylpyrrolidine (**2b**) afforded molecular ion peaks as doublets of equal intensity at *m/z* 511 and *m/z* 509, indicating the presence of bromine, and a strong peak at *m/z* 430 due to the loss of bromine. Similar peaks were observed for the pyrrolidides of the other fatty acids, differing by 14 amu depending on the chain-length. However, common to all of these pyrrolidides was a base peak at *m/z* 113 due to McLafferty rearrangment, a peak not so prominent at *m/z* 180, which corresponded to a double allylic fragmentation between C-6 and C-9 with the loss of bromine, and a strong doublet of equal intensity at *m/z* 258 and *m/z* 260 due to the same fragmentation with the bromine substituent intact (Figs. 1 and 2). This information limited the point of attachment of the bromine between C-1 and C-7.

Catalytic hydrogenation (PtO_2) of the brominated methyl esters yielded the corresponding saturated fatty acids. For example, 6-bromo-5,9-tetracosadienoic acid afforded tetracosanoic acid, 6-bromo-23-methyl-5,9-tetracosadienoic acid afforded 23-methyltetracosanoic acid, etc. It was then possible to calculate the corresponding ECL values for these methyl esters, and to locate the branching in the original molecules. For example, 23-methyltetracosanoic acid afforded an ECL value of 24.66 and 24-methylpentacosanoic acid, resulting from the hydrogenation of 6-bromo-24-methyl-5,9-pentacosadienoic acid, afforded an ECL value of 25.60. Therefore, the methyl branching in the original fatty acids is located at the penultimate carbon, corresponding to the *iso*-isomer. This methyl substitution was confirmed by GC co-injection with authentic samples.

The exact location of the bromine substituent was confirmed by deuteration of the pyrrolidides utilizing Wilkinson's catalyst [$RhCl(PPh_3)_3$] in methanol in order to avoid deuterium scrambling (Fig. 1). Five deuteriums were incorporated into the brominated fatty acid pyrrolidides. In all of the deuterated fatty acid pyrrolidides, a difference of 15 amu was observed between C-4 and C-5, between C-8 and C-9, and between C-9 and C-10, indicating the addition of deuterium atoms to the double bonds. Moreover,

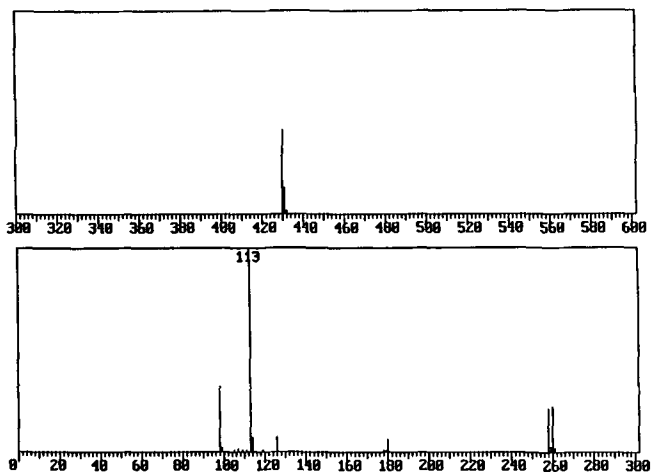

FIG. 2. Partial mass spectrum of the N-(5*E*,9*Z*)-6-bromopentacosa-5,9-dienoylpyrrolidine.

a difference of 16 amu was detected between C-5 (*m/z* 155) and C-6 (*m/z* 171), clearly demonstrating an additional replacement of bromine with deuterium at C-6 (1).

The GC/FTIR spectra of the original brominated fatty acid methyl esters displayed absorption peaks at 3018, 2993, 2956, 2878, 1739, 1592, 1453, 1264 and 1123 cm^{-1}. No peak at 980–968 cm^{-1} was observed, indicating *cis* rather than *trans* unsaturation for the Δ9 double bond, as is usual for demospongic acids (11). The stereochemistry at the Δ5 double bond was previously established as *E* by a microscale metal-halogen exchange reaction followed by protonolysis (2).

The sterols from *Agelas* sp. were also isolated, using reverse-phase high-performance liquid chromatography for the final separation. The particular sterols were characterized by 300 MHz ^1H NMR, MS and by comparison with authentic samples. The following sterols were characterized: cholesta-5,7,22-trien-3β-ol (9%), cholesta-5,7-dien-3β-ol (8%), 24-methylcholesta-5,7,22-trien-3β-ol (39%), 24-methylcholesta-5,7-dien-3β-ol (6%) and 24-ethylcholesta-5,7,22-trien-3β-ol (38%). For most of these sterols, the ^1H NMR spectrum showed the C-18 and C-19 angular methyl signals at δ 0.63 and 0.94 ppm, respectively, and two ring-olefinic signals deshielded to δ 5.38 and 5.58 ppm, corresponding to the Δ5,7 protons. It was particularly striking that neither cholesterol nor 24-ethylcholesterol was present in this sponge, as they are common sterols in sponges. This sponge seems to prefer Δ5,7 sterols. Whether these findings bear any connection with the unprecedented brominated phospholipids isolated in this work is just a matter of speculation at this point.

In this paper we have expanded present knowledge on the occurrence of brominated phospholipid fatty acids in marine sponges. Four new acids have been identified. As to their origin, we can only speculate but it is possible that they originated by the action of a bromoperoxidase on their nonbrominated counterparts, which were also found in their sponge (12). In fact, the biosynthesis of the 6-bromo-5,9-hexacosadienoic acid (**3a**) was previously investigated by Lam *et al.* (2) utilizing radiolabeled precursors, and they concluded that the bromination was the terminal step in the biosynthesis of these unusual acids. Based on the data available in the literature, we believe that the biosynthesis of the brominated acids follows the biosynthetic route shown below, as an example, for the 6-bromo-23-methyl-5,9-tetracosadienoic acid (**2b**).

i-15:0 → i-25:0 → i-Δ^5-and Δ^9-25:1 → i-$\Delta^{5,9}$-25:2 → i-6-Br-$\Delta^{5,9}$-25:2

Work is in progress trying to elucidate the origin of these unusual fatty acids in sponges.

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The Steryl Ester and Phospholipid Fatty Acids of the Sponge *Agelas conifera* from the Colombian Caribbean

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The steryl ester and phospholipid fractions of the marine sponge *Agelas conifera* were isolated and analyzed. The fatty acyl components of the steryl ester and phospholipid fractions as determined by gas chromatography and gas chromatography/mass spectrometry were very similar and consisted of 56.8 and 62.7% of C_{14}–C_{20} acids (normal; branched, especially *iso* and *anteiso*; and monounsaturated, particularly Δ^9 and Δ^{11} acids) and of 43.1 and 35.5% of C_{24}–C_{26} acids ($\Delta^{5,9}$ diunsaturated acids), respectively. The major constituent fatty acids detected were 13-methyltetradecanoic, *n*-hexadecanoic, 10-methylhexadecanoic, 11-octadecenoic, 12-methyloctadecanoic, 5,9-pentacosadienoic and 5,9-hexacosadienoic acids. The phospholipids isolated were identified as phosphatidylcholine (37%), phosphatidylserine (34%), phosphatidylethanolamine (16%) and phosphatidylinositol (11%). The distribution of fatty acids within the phospholipid classes was also determined.
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Agelas (family Agelasidae) is a genus that had been classified in the past as belonging to the order Poeilosclerida, but based on similarities in free amino acid patterns between *Agelas* and some Axinellidae, *Agelas* was more recently reassigned to the order Axinellida (1). However, further information on other *Agelas* constituents would still be desirable to substantiate the correctness of the new classification. As part of our studies on the chemical composition of marine sponges occurring in Colombian coastal waters (2–4), we therefore turned our attention to *Agelas conifera*, a very abundant sponge in the Caribbean Sea.

In 1989, we reported the sterol composition of *A. conifera* (4) (Δ^0 + Δ^7 sterols) which showed great similarities with that of *A. oroides* (5), *A. mauritiana* (6) and *A. dispar* (7). Recently, the presence of bromopyrrole metabolites in *A. conifera* (8) was also reported, a characteristic which is also shared with some members of Axinellidae. In continuation of this work and of earlier work by others (9–11), we here report our findings on the steryl ester and phospholipid fatty acids of *A. conifera*.

MATERIALS AND METHODS

A. conifera was collected from a depth of 10–20 m at Santa Marta Bay on the Caribbean coast of Colombia on three different occasions during one year. Sponge specimens were immediately cleaned and frozen. Total lipids were extracted by treating the thawed sponges (570 g) at 0°C for 5 min with chloroform/methanol (1:1, vol/vol) (12) followed by filtering and washing the organic layer twice with water containing sodium chloride.

Isolation of steryl esters. A portion of the crude total lipid extract (10 g) was purified by silica gel column chromatography using benzene/ethyl acetate (10:2, vol/vol) as the mobile phase to yield 620 mg of a mixture of steryl esters.

Isolation and analysis of phospholipids. Another portion of the crude lipid extract (0.8 g) was subjected to column chromatography using silicic acid (100 mesh) treated with ammonium hydroxide according to the procedure of Privett *et al.* (13) to separate the phospholipids from the neutral lipids and glycolipids.

The phospholipids thus obtained were kept under nitrogen at −10°C in chloroform/methanol (1:1, vol/vol) containing 0.002% of butylated hydroxytoluene. Subsequently, the phospholipids were further separated into classes by thin-layer chromatography (TLC) using double development and chloroform/methanol/28% ammonium hydroxide (65:35:8, by vol) as solvent. Fractions were made visible with molybdenum blue spray reagent. Phospholipid classes were identified by cochromatography with standards, and by use of Dragendorff, ninhydrine and periodate-Schiff spray reagents (14). Individual phospholipid classes were isolated by preparative TLC using above solvent and rhodamine 6G as spray agent. Phospholipids classes were quantified by a spectrophotometric phosphorus assay using molybdenum blue reagent (14).

Analysis of fatty acids. Saponification of the steryl ester fraction and methanolic hydrogen chloride treatment of the total phospholipid extract and of individual phospholipid classes were used to prepare both methyl esters and *N*-acyl pyrrolidides (15) for gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS).

The methyl esters were analyzed by GC using a Hewlett-Packard (Avondale, PA) 5700A gas chromatograph equipped with a crosslinked OV-101 fused silica capillary column (25 m × 0.31 mm, i.d.) and a flame-ionization detector (FID). The temperature program was started at 170°C and raised at 2°C/min to 270°C. Flow rates of carrier and make-up gases were 1 mL/min and 30 mL/min, respectively. Detector and injector temperatures were kept at 250 and 300°C, respectively.

GC/MS analyses were performed on a Shimadzu (Kyoto, Japan) 9020 DF gas chromatograph/mass spectrometer using the GC conditions mentioned above. Mass spectra were obtained in the electron impact mode under the following conditions: temperature of the ion source, 250°C; electron energy, 70 eV; ionizing current, 60 mA; injection volumes, 0.1 μL. Helium was used as the carrier gas.

The *N*-acyl pyrrolidides were analyzed by GC/MS under the conditions used for the methyl esters, except that the column temperature program was started at 230°C and raised at 4°C/min to 290°C.

Fatty acids were identified by GC based on equivalent chain length (ECL) values and by GC/MS based on the mass spectra of the methyl esters and pyrrolidides. Quantitations were based on integrated GC/FID peak areas.

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Abbreviations: ECL, equivalent chain length; FID, flame-ionization detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography.

RESULTS AND DISCUSSION

The phospholipids identified in the *A. conifera* marine sponge were phosphatidylcholine (PC) at 37%, phosphatidylserine (PS) at 34%, phosphatidylethanolamine (PE) at 16%, phosphatidylinositol (PI) at 11%, and one unidentified phospholipid at 2%.

High percentages of PE and PS, and at times PC, seem to be characteristic of marine sponges (15–18). By contrast, PI, which is one of the major phospholipids in bacteria, occurs only in very small amounts in eukaryotes. However, PI was found in significant quantities in preparations of *A. conifera*, which suggests that the PI originates from bacteria which live in symbiosis with the sponge. Vacelet and Donadey (19), in studies done on the associations between sponges and bacteria, have in fact demonstrated the presence of intercellular bacteria in several species of the genus *Agelas*, although no specific information on *A. conifera* was provided. Thus, the phospholipids reported here are likely to reflect the composition of *A. conifera* containing symbiotic bacteria.

The fatty acyl compositions of the total phosholipids, of the steryl esters and of individual phospholipid classes, as determined by GC and GC/MS analysis of the methyl esters and pyrrolidides are shown in Table 1. The table lists 25 fatty acids, which were identified together with the ECL values of the methyl esters. Identifications were made based on ECL values and comparison with the ECL values of standards. In addition the mass spectra of the methyl esters provided molecular weight information and helped in assessing branching in the saturated esters. The mass spectra of the *N*-acyl pyrrolidides confirmed these identifications and also provided information which helped in localizing the positions of double bonds in most of the unsaturated acids. The spectral data were also compared with those of authentic reference compounds and with other published spectra (Environmental Protection Agency National Institutes of Health mass spectral library).

The fatty acid distributions in the steryl ester and in the phospholipid classes were found to be very similar. In both fractions there were normal chain saturated acids (C_{14}, C_{15}, C_{16}, C_{17}, C_{18} and C_{20}), branched chain and particularly *iso* and *anteiso* saturated acids (C_{15}, C_{16}, C_{17}, C_{18}, C_{19} and C_{20}), monounsaturated acids, *i.e.* mainly Δ^9 and Δ^{11} (C_{16}, C_{17}, C_{18}, C_{19} and C_{23}) and $\Delta^{5,9}$ normal chain diunsaturated acids (C_{24}, C_{25} and C_{26}). The diunsaturated compounds comprised about 43% of the total fatty acids in the steryl ester fraction and 35% in the phospholipid fraction; the monounsaturated fatty acids represent 15 and 17% in the steryl ester and in the phospholipid fractions, respectively, amounting to a total of 58 and 52% of unsaturated fatty acids in these two fractions. Of the remaining fatty acids, 20% correspond to saturated normal chain and 22% to saturated branched chain acids within the steryl esters, and 14% to saturated normal chain and 31% to saturated branched chain acids in the phospholipids.

It is important to point out the high percentage of fatty acids found in this sponge having an odd number of

TABLE 1

Steryl Ester and Phospholipid Fatty Acids of *Agelas conifera*[a]

Number	ECL	Fatty acid	Abundance (%)						
			Steryl ester fraction	Phospholipid fraction	PE	PC	PS	PI	Unknown phospholipid
1	13.60	12-Methyltridecanoic (*iso*-14:0)	—	0.5	—	1.1	—	—	—
2	14.00	Tetradecanoic (*n*-14:0)	3.2	2.5	4.5	5.8	—	15.1	14.7
3	14.61	13-Methyltetradecanoic (*iso*-15:0)	2.8	6.2	—	4.3	—	9.6	—
4	14.70	12-Methyltetradecanoic (*anteiso*-15:0)	1.5	2.6	—	2.4	—	3.6	—
5	15.00	Pentadecanoic (*n*-15:0)	0.6	0.8	2.1	2.6	—	5.7	7.2
6	15.66	14-Methylpentadecanoic (*iso*-16:0)	0.7	1.3	—	1.5	—	3.4	—
7	15.81	9-Hexadecenoic (Δ^9-16:1)	3.1	1.4	3.4	4.5	—	2.2	12.6
8	16.00	Hexadecanoic (*n*-16:0)	10.1	7.1	12.4	27.8	9.7	16.8	37.7
9	16.45	14-Methyl-9-hexadecenoic (Δ^9-*anteiso*-17:1)	3.5	4.3	—	3.0	—	—	—
10	16.43	10-Methylhexadecanoic (10-Me-16:0)	6.7	6.6	—	7.3	11.3	2.2	—
11	16.66	15-Methylhexadecanoic (*iso*-17:0)	1.7	2.1	—	2.6	—	3.0	—
12	16.73	14-Methylhexadecanoic (*anteiso*-17:0)	1.2	1.2	—	1.8	—	—	—
13	16.87	Heptadecenoic (17:1)	0.9	0.4	—	1.7	—	—	—
14	17.00	Heptadecanoic (*n*-17:0)	1.1	0.4	—	1.7	—	—	—
15	17.69	15-Methylheptadecanoic (*anteiso*-18:0)	1.1	0.5	3.2	3.6	—	—	11.4
16	17.78	11-Octadecenoic (Δ^{11}-18:1)	4.6	7.3	—	4.1	2.5	—	—
17	18.00	Octadecanoic (*n*-18:0)	4.1	2.6	4.6	8.8	4.5	13.6	16.4
18	18.44	12-Methyloctadecanoic (12-Me-19:0)	4.3	9.1	2.4	9.6	8.2	24.7	—
19	18.88	11-Nonadecenoic (Δ^{11}-19:1)	2.6	2.7	4.4	—	—	—	—
20	20.00	Eicosanoic (*n*-20:0)	1.2	0.7	—	—	—	—	—
21	20.44	?-Methyleicosanoic (Me-20:0)	1.8	1.2	—	—	4.0	—	—
22	22.86	11-Tricosaenoic (Δ^{11}-23:1)	—	1.2	—	—	—	—	—
23	23.50	5,9-Tetracosadienoic ($\Delta^{5,9}$-24:2)	5.6	3.2	5.4	—	4.4	—	—
24	24.50	5,9-Pentacosadienoic ($\Delta^{5,9}$-25:2)	17.0	13.6	19.8	2.2	17.4	—	—
25	25.27	Unidentified	—	1.2	—	—	18.2	—	—
26	25.50	5,9-Hexacosadienoic ($\Delta^{5,9}$-26:;2)	20.5	18.7	37.6	3.6	19.7	—	—

[a]Equivalent chain length (ECL) values are those of the methyl esters of these acids. Averages from the analyses of the three samples collected over one year (seasonal variations were not observed). A dash indicates an abundance of less than 0.4%. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

carbon atoms (41% in the steryl ester fraction and 43% in the phospholipid fraction), which cannot be attributed only to associated bacteria.

From the distribution of fatty acids within the phospholipid classes it is apparent (Table 1) that short-chain fatty acids occur mainly in PC, PI and in the unidentified phospholipid, with tetradecanoic, hexadecanoic, 12-methyloctadecanoic and octadecanoic acids being the major components. The long-chain $\Delta^{5,9}$ fatty acids are mainly found in PE and PS. These results are in general agreement with the analyses done on other sponges (16–18) which have consistently shown that demospongic acids are primarily found in phospholipids containing a free amino group. Our results also indicate that bacterial type fatty acids predominate in PI, PC and in the unknown phospholipid.

According to Bergquist *et al.* (10), only the broad descriptive parameters of the fatty acid mixtures (straight and branched, even and odd, unsaturated and long-chain fatty acids) are relevant to taxonomic considerations. Thus, the fatty acid profile of *A. conifera* is characterized by a high percentage (35%) of long-chain fatty acids with a predominance in even-carbon numbered acids, medium levels (36%) of branched fatty acids with a prodominance in odd-carbon numbered acids, and high levels (63%) of straight chain acids with a predominance of even-carbon numbered compounds. Except for the high odd and branched acid content, these results seem to agree well with the fatty acid profile reported for some species from the other two Axinellida families (10). In contrast, it is important to mention the low content (11%) of long-chain fatty acids reported by Carballeira *et al.* (20) for the sponge *A. dispar.*

Although the results seem to indicate homogeneity in the fatty acid patterns in the Axinellida families, it is also clear that a number of additional *Agelas* species need to be analyzed as to their fatty acid composition in order to obtain a more complete picture of the fatty acid patterns in the Agelasidae family.

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Identification of Two Novel Dihydroxysterols from *Pavlova*

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***Pavlova gyrans* and *P. lutheri* were cultured, and the dihydroxysterols were isolated from the free sterol and the polar sterol fractions. Four dihydroxysterols were detected in amounts greater than 1% of total sterol by gas chromatography and were analyzed by gas chromatography/mass spectrometry. The two principal sterols were isolated by chromatography on alumina followed by hydrophobic Sephadex column chromatography. The two sterols appeared to differ by having either a methyl or an ethyl group at C-24; they were termed "methylpavlovol" and "ethylpavlovol." Analysis by 400 MHz nuclear magnetic resonance showed that methylpavlovol is 4α,24β-dimethylcholestan-3β,4β-diol and ethylpavlovol is 4α-methyl,24β-ethylcholestan-3β,4β-diol. The 4α-methyl,4β-hydroxy configuration has not been observed previously in a natural sterol. Dihydroxysterols make up approximately one-third of the total sterols in these *Pavlova* species. Neither the biosynthetic origin of these dihydroxysterols nor their role in the biochemistry of *Pavlova* is known.**

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Bivalves feed on phytoplankton and other particulate matter. Attempts to culture bivalves, such as oysters and clams, have utilized various phytoplankton as food. Supplementation with other factors, including carbohydrates, proteins, lipids and polyunsaturated fatty acids, as well as sterols, has also been shown to be important in the oyster's diet (1,2). Species of *Pavlova* have been included frequently in oyster diets (3). *Pavlova gyrans* and *P. lutheri* were found to have an unusual sterol composition and to contain C_{28} and C_{29} Δ^5-sterols, 4α-methyl sterols with a saturated nucleus and a group of unknown sterols apparently bearing two hydroxyl groups (4). The sterol content of these phytoplankton is unusually high (7-9 mg/g dry wt) compared to that of most other phytoplankton we have examined (Patterson, G.W., and Wikfors, G.H., unpublished). Sterols are found as free sterols, steryl esters or steryl glycosides in plants (5), but in algae most sterols occur in the free form (Patterson, G.W., and Wikfors, G.H., unpublished). *P. gyrans* is unusual in that about one-third of its sterols is found in the polar fraction where the principal components were poriferasteryl glucoside and ethylpavlovol monoglucoside (6). The objective of this study was to isolate, characterize and identify the major dihydroxysterols of *P. gyrans* and *P. lutheri*.

MATERIALS AND METHODS

P. gyrans Butcher, strain designation "93" and *P. lutheri* "MONO" were obtained from the Milford laboratory culture collection (Milford, CT) and were cultured axenically in enriched seawater medium "E" formulation and harvested in stationary phase as described previously (4). The lipid fraction was obtained from lyophilized algae by $CHCl_3/CH_3OH$ extraction in a Soxhlet apparatus. Sterols obtained from the free sterol and the polar sterol fractions after fractionation of the total lipid on Biosil A (4) contained dihydroxysterols at 25-50% of the total sterols, with the largest percentages occurring in the polar sterol fraction. The free sterol fraction and free sterols generated from the polar sterol fraction by acid hydrolysis were pooled and chromatographed by alumina column chromatography to separate dihydroxy and monohydroxy sterols. Dihydroxy sterols were further fractionated by lipophilic Sephadex chromatography (7) which produced samples of a C_{29} dihydroxysterol (methylpavlovol) and a C_{30} dihydroxysterol (ethylpavlovol) in amounts permitting analysis by 400 MHz nuclear magnetic resonance (NMR). ^{13}C and ^1H NMR spectra were obtained with a Bruker AM-400 (Karlsruhe, Germany) spectrometer operating at frequencies of 400.179 MHz for ^1H and 100.619 MHz for ^{13}C using tetramethylsilane as an internal standard (8). Specific pulse sequences used included DEPT-135 (9), NOESY (10), COSY (11) and C-H correlation (12) experiments, which were conducted as referenced. Electron impact mass spectra were measured at 70eV and an ionization temperature of 150°C. Data were recorded with an Incos Data System (Incos Finnigan Corp., San Jose, CA).

RESULTS AND DISCUSSION

In *P. gyrans*, four dihydroxysterols were detected by capillary gas chromatography in the late-eluting fractions from alumina chromatography. These compounds had retention times relative to cholesterol of 2.06, 2.13, 2.22 and 2.52 in capillary gas chromatography on SPB-1. *P. lutheri* contained each of these compounds except 2.13. Compounds 2.06 and 2.52 eluted slightly earlier and were separated from compounds 2.13 and 2.22 on alumina chromatography, but could not be separated from each other by this method. Gas chromatography/mass spectrometry (GC/MS) was performed on the mixture of compounds 2.06 and 2.52. The major peaks observed are listed in Table 1. The mass spectra indicate the presence of C_{29} and C_{30} homologs which are saturated sterols having two hydroxyl groups. Apparently these are sterols with C_9 and C_{10} side chains possessing an "extra" carbon on the nucleus. This extra carbon is probably at C-4, as the strong M-15 peak characteristic of 14-methyl sterols is not present. Acetylation of these sterols yielded only monoacetates, suggesting the presence of a tertiary hydroxyl.

Volkman *et al.* (13) had noted that a dihydroxysterol from *P. salina* dehydrated in the presence of BF_3/methanol to produce a C_{29} methylstanone, suggesting that the compound in question was a 3,4-dihydroxy

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Abbreviations: GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance.

TABLE 1

Mass Spectral Data for the Saturated Dihydroxysterols of *Pavlova*

Fragmentation	Sterol[a]	
	Methylpavlovol	Ethylpavlovol
$[M]^+$	432 (18)	446 (16)
$[M - CH_3]^+$	417 (57)	431 (50)
$[M - H_2O]^+$	414 (8)	428 (7)
$[M - 33]^+$	399 (19)	413 (18)
$[M - 59]^+$	373 (32)	387 (31)
$[M - 75]^+$	357 (44)	371 (41)
Base peak	95 (100)	95 (100)

[a]Reported as *m/z* (relative intensity).

4-methyl sterol. The homologous dihydroxysterols from *P. gyrans*, which we have termed "methylpavlovol" and "ethylpavlovol," were separated by column chromatography on lipophilic Sephadex and analyzed by ^{13}C and ^1H NMR spectrometry. The assignments for ethylpavlovol were readily made based on the results obtained by various NMR techniques and by comparison with literature values (14,15). Two hydroxy-bearing signals were detected from ethylpavlovol (2.52), at 75.65 ppm and 74.17 ppm, but only the signal at 75.65 ppm was detected in a DEPT-135 experiment. The C-3 hydroxy, arising from a methine carbon, gives the signal at 75.65 ppm and the 74.17 ppm signal arises from a quaternary carbon (C-4). The chemical shift of C-3 at 75.65 ppm is consistent with the presence of a methyl at C-4 (C-3 of 4α-methylcholestan-3β-ol resonates at 76.6 ppm and the C-3 of 4β-methylcholestan-3β-ol at 74.1 ppm) (16). There are also three singlet methyl signals in the ^1H NMR spectrum. 1D NOE difference experiments, with irradiation at H-30 or H-19, revealed no detectable interaction between these two methyls. A NOESY experiment (650 ms mixing time) did not detect a crosspeak between H-30 and H-19, but did detect an H-3 *vs.* H-30 crosspeak. Thus, the 4α-methyl-4β-hydroxy structure is preferred over the 4β-methyl-4α-hydroxy structure. Coincidentally, molecular mechanics calculations showed that the 4α-methyl-3β,4β diol is more stable by 1.48 kcal/mole than the 4β-methyl-3β,4α-diol structure. ^1H NMR analysis of the methyl signals showed the orientation of the C-24 ethyl to be β as it is in most algae (17). The structure of ethylpavlovol, therefore, is 4α-methyl-24β-ethylcholestan-3β,4β-diol (Fig. 1).

^{13}C and ^1H NMR analyses of methylpavlovol gave results similar to those of ethylpavlovol (Table 2) with the exception of the side chain signals. These signals correspond to those of a C-24β(24S) methyl by the closer fit of the C-20, C-23, C-24, C-25, C-26 and C-27 peaks (36.21, 30.65, 39.13, 31.52, 17.64 and 20.51 ppm, respectively) to those reported for the 24β/S side chain (36.3, 30.7, 39.2, 31.5, 17.7, 20.6 ppm) (16), than to those reported for the

TABLE 2

NMR Results for Methylpavlovol and Ethylpavlovol[a]

Carbon	^{13}C Chemical shifts		^1H Chemical shifts[b]	
	Methylpavlovol	Ethylpavlovol	Methylpavlovol	Ethylpavlovol
1	36.68	36.61		1.74, 1.69
2	27.21	27.17		1.69
3	75.69	75.65	3.249	3.250
4	74.18	74.17		
5	53.00	52.92		0.90
6	20.62	20.60		1.75
7	32.52	32.48		1.80, 0.87
8	34.96	34.90		1.37, 1.32
9	55.67	55.59		0.59
10	36.26	36.22		
11	20.64	20.60		1.43, 1.28
12	40.02	39.97		1.96, 1.10
13	42.58	42.53		
14	56.66	56.61		0.94
15	24.21	24.19		1.55, 1.06
16	28.25	28.28		1.83
17	56.16	56.12		1.09
18	12.10	12.07	0.643	0.646
19	13.99	13.97	1.006	1.009
20	36.21	36.28		1.36
21	18.86	18.77	0.899	0.906
22	33.76	33.89		1.36, 0.96
23	30.65	26.37		1.29
24	39.13	46.05		0.92
25	31.52	28.93		1.67
26	17.64	19.60	0.779	0.828
27	20.51	18.97	0.771	0.807
28	15.48	23.00	0.852	1.323, 1.16
29		12.32		0.851
30	25.37	25.36	1.225	1.229

[a]Results given in ppm. NMR, nuclear magnetic resonance.
[b]Protons 3, 18, 19, 21, 26, 27, 28 (methylpavlovol), 29 (ethylpavlovol) and 30 were measured directly from the 1D NMR spectra. The remainder were obtained from C–H correlation spectra and are reported to fewer significant figures.

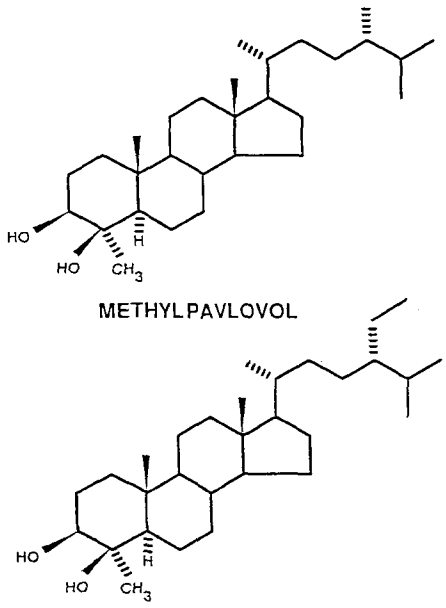

FIG. 1. The structures of methylpavlovol (4α,24β-dimethylcholestan-3β,4β-diol) and ethylpavlovol (4α-methyl-24β-ethylcholestan-3β,4β-diol) from *Pavlova gyrans* and *P. lutheri*.

24α/R side chain (36.0, 30.4, 38.9, 32.5, 20.3, 18.3 ppm) (16). Methylpavlovol and ethylpavlovol differ from the stanols 4α-methylergostanol and 4α-methylporiferastanol, already identified in *Pavlova* (4), *Prototheca* and *Dictyostelium* (15) only by the presence of the 4β-hydroxyl group. The 4α-methyl-4β-hydroxy grouping has not been observed previously to occur on a natural sterol. The biosynthetic relationship between the dihydroxysterols and the other sterols of *Pavlova* has not been investigated. Work is continuing to identify two other C_{29} dihydroxysterols in *P. gyrans*.

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Age-Related Accumulation of Phosphatidylcholine Hydroperoxide in Cultured Human Diploid Cells and Its Prevention by α-Tocopherol

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The levels of phosphatidylcholine hydroperoxide in serially cultured human fetal diploid fibroblasts at various population doubling levels were determined by high-performance liquid chromatography combined with chemiluminescence detection. This methodology utilizes a mixture of cytochrome *c* and luminol as post-column hydroperoxide group specific luminescent reagents. The cellular hydroperoxide content increased with age from 0.34 to 27.72 pmol/10^6 cells. At the end of the cells' *in vitro* lifespan (51st population doubling level), the hydroperoxide content per 10^6 cells reached about 80 times the level found in cells of the 20th population doubling level. Supplementation of exogenous α-tocopherol to the culture medium prevented hydroperoxide accumulation, but did not extend the lifespan *in vitro*. The results indicate that substantial intracellular phospholipid hydroperoxide accumulation occurred in the course of aging of human fetal diploid fibroblasts.

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Peroxidation of membrane lipids has been suggested as one of the basic deteriorative reactions in cellular senescence (1–4). Since Harman (1) proposed the "free radical theory of aging" in 1956, numerous studies have been undertaken to determine the relationship between the aging process and lipid peroxidation. Yet, direct evidence for such a relationship is still lacking. In previous studies, the link between aging and tissue lipid peroxidation could not be sufficiently assessed because the thiobarbituric acid (TBA) test commonly employed in such experiments is not specific for lipid hydroperoxides (5–7). Measurement of expired hydrocarbon gas from rats (8) and lipofuscin pigment measurements in rat tissues (9) and cultured diploid cells (10) also have been used as a lipid hydroperoxidation index relative to aging, but these methods rely on the measurement of secondary oxidation products. To assess the degree of lipid hydroperoxidation in biological membranes, the direct detection of primary peroxidation products, such as phospholipid hydroperoxides, seems to be advantageous. Changes in intracellular Na^+ and K^+ content, membrane fluidity and membrane lipid transition temperature following aging have been reported (11,12). Because phospholipids are a major constituent of membranes and contribute to membrane function, direct measurement of phospholipid hydroperoxide seems to be an important approach to assess membrane lipid peroxidation.

We have previously developed a sensitive and selective assay for phospholipid hydroperoxides using high-performance liquid chromatography (HPLC) combined with chemiluminescence (CL) detection (13–15). This CL–HPLC method has been successfully applied to the estimation of phospholipid hydroperoxide levels in human blood plasma (14,16), serum lipoproteins (17), liver of carbon tetrachloride intoxicated rats (18) and hepatocarcinogenesis in rats on choline-deficient and ethionine-enriched diets (19). The CL–HPLC method has also been employed in other laboratories to determine hydroperoxides (20–22).

In the present study, we investigated the age-dependent changes of phosphatidylcholine hydroperoxide (PCOOH) levels in cultured human fetal diploid cells as determined by the CL–HPLC method. The effect of α-tocopherol, a potent biological antioxidant, on PCOOH content and cellular lifespan was examined.

MATERIALS AND METHODS

Cell culture. Normal human diploid fibroblasts (HE-1) were established by Dr. Sato, Institute of Osaka Oriental Medicine (Osaka, Japan); their final population doubling level (PDL) is the 51st. The cells were serially cultured in 20 mL of Eagle's minimum essential medium that contained 10% fetal calf serum. A single lot of fetal calf serum (Whittaker M.A. Bioproducts, Rockville, MD) was used throughout the experiments. Cultures were maintained in 250-mL Falcon flasks at 37°C in an atmosphere of 95% air and 5% carbon dioxide. The cells were subcultured weekly at a 1:2 split ratio using 0.25% trypsin containing 0.01% butylated hydroxytoluene as an antioxidant.

α-Tocopherol treatment. d,l-α-Tocopherol was solubilized by mixing it into growth medium using polyethylene glycol No. 400 (Tokyo Kasei Kogyo Co., Tokyo, Japan). *d,l-α*-Tocopherol (Eisai Co., Tokyo, Japan) was added to the medium at 0.1, 1 and 10 μg per mL after the 42nd PDL to examine its antioxidant effect. The final concentration of polyethylene glycol in the medium was 0.1% throughout all experiments using a three-graded amount of exogenous α-tocopherol. α-Tocopherol content was determined by the fluorescence–HPLC method (23) on the total lipids extracted as described below. Cells were counted with a Coulter Counter ZBI (Coulter Electronics, Hialeah, FL).

Lipid extraction. Cells were harvested by treatment with 0.25% trypsin containing 0.01% butylated hydroxytoluene and centrifuged at 1,000 rpm for 5 min. Two mL of 0.15 M sodium chloride containing 0.002% butylated hydroxytoluene was added to 5×10^6 cells, and the mixture was homogenized in a Teflon-glass homogenizer under ice cooling. The homogenate obtained was added to 5 mL of chloroform/methanol (2:1, vol/vol) (24,25) and mixed vigorously for 1 min. The mixture was centrifuged at 3,000 rpm for 10 min. The lower chloroform layer containing total lipid was dried over anhydrous sodium sulfate, the solvent was removed in a rotary evaporator and the residue was dried under a nitrogen stream. The total lipid obtained was dissolved in 100 μL of a mixture of

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Abbreviations: CL, chemiluminescence; HPLC, high-performance liquid chromatography; PCOOH, phosphatidylcholine hydroperoxide; PDL, population doubling level.

chloroform/methanol (2:1, vol/vol), and a 20 μL portion was subjected to the hydroperoxide assay by CL-HPLC (18,19).

CL-HPLC analysis. The CL-HPLC system and experimental conditions for measuring PCOOH in the total lipids prepared from human diploid cells were essentially as described previously by Miyazawa *et al.* (14,16-18) and Yoshida *et al.* (19). Analytical conditions were as follows: the HPLC column was a JASCO Finepak SIL (5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Tokyo, Japan), the mobile phase was a mixture of chloroform/methanol/water (1:9:0.1, by vol), and the flow rate was 1.1 mL/min using a JASCO 880-PU pump. After passing through a JASCO 875-UV detector set at 234 nm to monitor conjugated diene, the column eluant was mixed with a luminescent reagent at a post-column mixing joint (Y type; Kyowa Seimitsu, Tokyo, Japan). The luminescent reagent was prepared by dissolving 10 μg/mL of cytochrome *c* (from horse heart, type VI; Sigma Chemical Co., St. Louis, MO) and 1 μg/mL of luminol (3-aminophthaloyl hydrazine; Wako Pure Chemical Co., Osaka, Japan) in 50 mM borate buffer (pH 9.3) and was pumped at 1.0 mL/min with a JASCO 880-PU pump. The CL generated by reacting the hydroperoxide with the luminescent reagent was measured with a CLD-100 CL detector (Tohoku Electronic Industries Co., Sendai, Japan) or a JASCO 825-CL detector. Calibration of PCOOH was done with authentic PCOOH, which was prepared from egg yolk phosphatidylcholine (98.6%, Nippon Oil & Fats Co., Tokyo, Japan) by photooxidation as described previously (13-15).

RESULTS AND DISCUSSION

Figure 1 shows the CL chromatogram of PCOOH when the total lipids extracted from cultured human diploid cells (48th PDL) were subjected to the CL-HPLC assay. PCOOH (R_t = 13 min) was eluted and measured as a single sharp peak by CL detection. This PCOOH peak has the same retention time as does authentic PCOOH. The retention time was also confirmed by cochromatography with the authentic PCOOH. By treatment of total lipid with sodium borohydride, the chemiluminescence peak ascribed to PCOOH disappeared as PCOOH was reduced to the corresponding nonluminescent hydroxyl phosphatidylcholine. Other CL peaks detected near 2 and 4 min were tentatively assigned to mixtures of phosphatidylethanolamine hydroperoxide, phosphatidylserine hydroperoxide and neutral lipid hydroperoxides. These CL peaks also disappeared after sodium borohydride treatment.

Figure 2 shows the content of PCOOH per 10^6 cells as a function of PDL. The cellular content of PCOOH was increased with advancing serial subcultures. The cells at the 20th PDL corresponded to the middle of the active multiplication period (phase II) as defined by Hayflick and Moorhead (26). The cellular PCOOH content at all PDLs was compared with that of the 20th PDL (figures in parentheses of Fig. 2). From the 20th PDL to the 47th PDL, the PCOOH content increased less than threefold, but then PCOOH levels markedly increased after the 48th PDL. The PCOOH level of the 48th PDL was about thirteen times that of the 20th PDL, the 50th PDL fifty times that and the 51st PDL eighty times that of the 20th PDL.

Table 1 shows the effect of α-tocopherol addition to the culture medium on cell numbers, and α-tocopherol and PCOOH contents in cultured cells. The cell numbers of control (not treated with α-tocopherol) showed a decrease

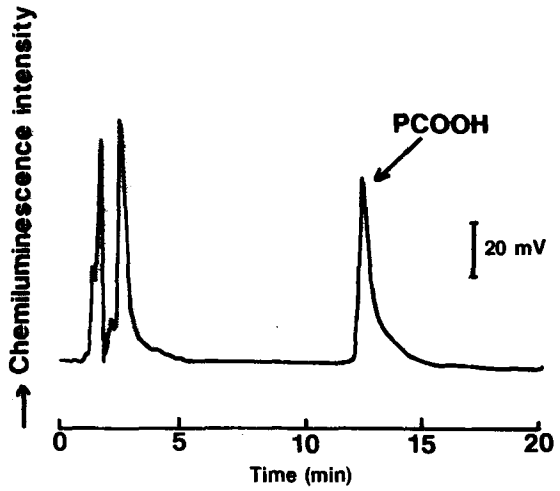

FIG. 1. Chemiluminescence chromatogram of phosphatidylcholine hydroperoxide (PCOOH) when total lipid extracted from cultured human diploid cells (48th population doubling level) was subjected to chemiluminescence-high-performance liquid chromatography assay. The PCOOH concentration detected on the chemiluminescent chromatogram corresponded to 4.5 pmol of PCOOH/10^6 cells.

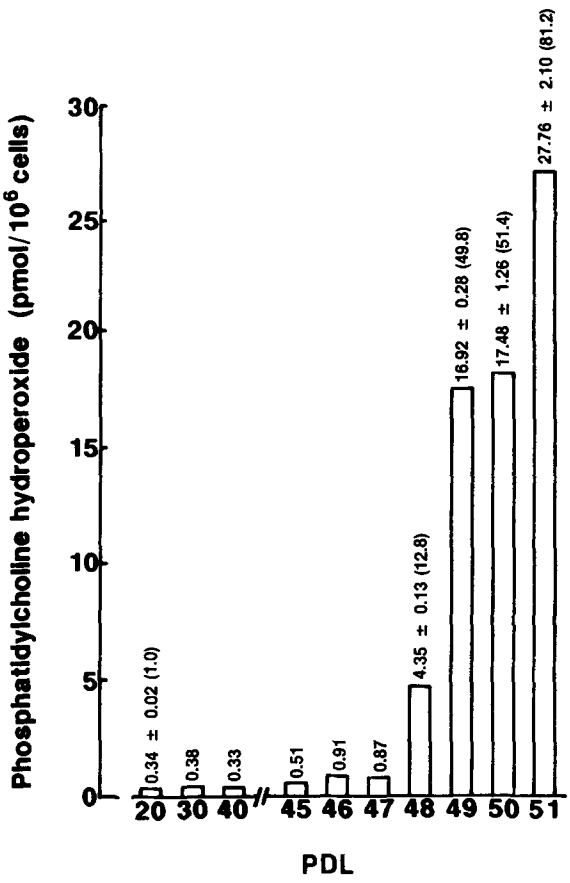

FIG. 2. Changes in the phosphatidylcholine hydroperoxide content per 10^6 cells as a function of population doubling level (PDL). Values are the mean of three experiments and SD. Figure in parentheses is the value relative to young cells (20th PDL).

TABLE 1

Effect of *d,l*-α-Tocopherol on Phosphatidylcholine Hydroperoxide (PCOOH) Content of Cultured Human Fetal Diploid Cells[a]

Population doubling level (PDL)	Cell numbers (× 10^6 cells/flask)				α-Tocopherol (ng/10^6 cells)				PCOOH content (pmol/10^6 cells)			
	0	0.1	1	10[b]	0	0.1	1	10[b]	0	0.1	1	10[b]
45	28	32	27	22	<1	2	24	780	0.5	0.3	0.3	0.4
46	31	32	28	16	<1	2	21	870	0.9	0.2	0.2	0.5
47	32	29	25	8	<1	3	24	1600	0.9	0.9	0.3	0.9
48	29	29	23	6	<1	2	20	1400	4.4	0.7	0.5	3.2
49	24	23	17	3	<1	3	21	1500	16.9	2.1	0.7	18.7
50	20	19	14	—[c]	<1	2	20	—[c]	17.4	2.2	0.7	—[c]
51	10	10	8	—[c]	<1	2	24	—[c]	27.7	8.8	0.8	—[c]

[a]Values represent the average of three experiments.
[b]*d,l*-α-Tocopherol (0, 0.1, 1 and 10 μg/mL medium) was added to the medium.
[c]Not determined.

after the 49th PDL. Supplementation with 1 or 10 μg of α-tocopherol/mL medium decreased cell numbers more rapidly when compared to controls. α-Tocopherol levels in cultured cells increased in relation to the amount of exogenous α-tocopherol added to the medium. About 1.0–8.6% of α-tocopherol exogenously added to the growth medium was incorporated into the cells (3.2% at 0.1 μg/mL, 3.2% at 1 μg/mL and 8.6% at 10 μg/mL at the 45th PDL; 3.5% at 0.2 μg/mL, 1.8% at 1 μg/mL and 2.2% at 10 μg/mL at the 49th PDL; 1.0% at 0.1 μg/mL and 1.0% at 1 μg/mL at the 51st PDL). Sakagami and Yamada (27) had reported that 2.0–4.0% of α-tocopherol added to the medium was incorporated into the cells.

Accumulation of cellular PCOOH with serial subculturing was also related to a decrease in the cellular proliferation rate. According to the original concept of Hayflick and Moorhead (26) and Hayflick (28), the limited *in vitro* lifespan of cells was characteristic of phase III, which is the period of an apparent decrease in the rate of cell proliferation accompanied by morphological, biochemical and cytological changes. The present results suggest that cellular phospholipid hydroperoxidation occurred in phase III. The lifespan of cultured diploid cells is known to be reduced under hyperbaric oxygen (29) and is extended under lower oxygen concentrations (30). These results also suggested that oxidative deterioration of membrane lipids associated with membrane phospholipid hydroperoxidation contributes to cellular aging.

Supplementation of 0.1 or 1 μg/mL of α-tocopherol to the growth medium effectively prevented PCOOH accumulation for all PDL as compared to controls. A higher PCOOH accumulation was observed when 10 μg/mL of α-tocopherol was added to the medium as compared to the addition of 0.1 or 1 μg/mL of α-tocopherol. α-Tocopherol is commonly considered a potent antioxidant, but in this case of lipid autoxidation, α-tocopherol in high concentrations showed a prooxidant effect (31,32). In cultured cells, an optimum α-tocopherol concentration that can effectively prevent membrane lipid hydroperoxidation should be maintained. Although supplementation of small amounts of α-tocopherol (0.1 and 1 μg/mL medium) prevents the accumulation of PCOOH, it is possible as previously reported (27,33), that a lifespan extension could not be observed in the present study.

We recently found that PCOOH accumulated significantly in the liver and brain of rats with age (34). The PCOOH content of *Drosophila melanogaster* flies was reciprocally proportional to their lifespan (35,36).

In conclusion, in the present study accumulation of cellular PCOOH was clearly shown to be associated with an increase in cellular PDLs. Supplementation with 0.1 or 1 μg/mL medium of α-tocopherol prevented cellular phospholipid hydroperoxide accumulation, but did not extend *in vitro* lifespan.

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ARTICLE

Formation of Octadecadienoate Dimers by Soybean Lipoxygenases

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The aim of this investigation was to determine whether the regioselectivity found for lipoxygenases in the formation of fatty acid hydroperoxides from linoleic acid is reflected in the formation of dimeric products in secondary reactions involving linoleic acid, product hydroperoxide and lipoxygenase. A method was therefore developed for the separation and identification of dimers formed by fusion of two linoleic acid radicals or a linoleic acid radical and linoleate. The method includes solid-phase extraction, preparative separation of products by thin-layer chromatography, derivatization to the corresponding fully hydrogenated methyl esters and capillary gas chromatography (GC) coupled with electron impact mass spectrometry. We present evidence that the formation of octadecadienoate dimers, during the secondary reaction of soybean lipoxygenase-1 or lipoxygenase-3, is a nonenzymic process that can be envisaged by nonspecific association of intermediate fatty acid radicals (L•) that have dissociated from the enzyme. We could show that the relative amounts of different octadecadienoate dimers formed remain unaltered, regardless of pH and type of soybean isoenzyme used. Quantitative analysis by GC showed that under the reaction conditions used, the formation of dimers branching at the 13-position is preferred.

Lipids 28, 779–782 (1993).

Lipoxygenases (LOX; linoleate:oxygen oxidoreductases, EC 1.13.11.12) comprise a class of enzymes that catalyze the regio- and stereoselective dioxygenation of polyunsaturated fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems. The products are chiral *Z,E*-conjugated fatty acid hydroperoxides. In mammalian tissues, LOX are involved in the initial steps of the biosynthesis from arachidonic acid of physiologically active compounds, including leukotrienes and lipoxins (1). In plants, these enzymes have been suggested to play a role in germination and possibly in the formation of anti-pathogens (2,3). Furthermore, LOX has been shown to be involved in the biosynthesis of 12-oxo-phytodienoic acid (4), a precursor of jasmonic acid (5). Considerable attention has been paid to the plant LOX, and in particular the soybean isoenzymes have been thoroughly investigated.

Three distinct types of LOX isoenzymes can be purified from soybeans, usually designated as LOX-1, LOX-2 and LOX-3. These isoenzymes differ with respect to isoelectric point, reactivity toward substrates, and optimal reaction conditions. The product of the aerobic reaction of LOX-1 with linoleic acid is almost exclusively 13*S*-hydroperoxy-9*Z*, 11*E*-octadecadienoic acid, while with LOX-2 and LOX-3, regio- and stereospecificity are less pronounced. Privett *et al.* (6) have reported that in addition to this primary reaction, lipoxygenases are also capable of performing a secondary reaction during which, among others, polymeric compounds are formed. Garssen *et al.* (7) were the first to identify the mechanism of this secondary reaction in which dimeric compounds, oxodienoic acids and *n*-pentane are formed *via* a coupled enzymic reaction of linoleic acid and product hydroperoxide. LOX-1 shows this secondary reaction only under anaerobic conditions (7). With LOX-3 it can occur both under aerobic and anaerobic conditions whereas LOX-2 shows very little formation of such products under any condition (8). In previous studies only limited information on the structures of the linoleic acid radical-derived dimeric products that are formed during the secondary reaction of lipoxygenases with linoleic acid could be obtained (9,10). This was mainly due to the lack of suitable methods for the separation and analysis of the various positional isomers. The aim of this investigation was to determine whether the regiospecificity found for soybean LOX in the formation of fatty acid hydroperoxides is reflected in the formation of dimeric products. To this end, the positional isomers of the octadecadienoate dimers were completely separated and analyzed.

MATERIALS AND METHODS

LOX-1 and LOX-3 were isolated from soybeans (11,12). Specific activities, as measured polarographically using a thermostated Clark-type oxygen electrode (Hansatec, King's Lynn, United Kingdom) were 230 μmol min^{-1} for LOX-1 (pH 9.0) and 25 μmol min^{-1} mg^{-1} for LOX-3 (pH 6.6). Regiospecificity of the different isoenzymes was determined by performing small-scale (10 mL) incubations with linoleic acid (100 μM) dissolved in oxygen-saturated 0.1 M sodium borate (pH 9.0) or 0.1 M sodium phosphate buffer (pH 6.6) at 25°C. After acidification of the reaction mixtures to pH 3.0 followed by extraction with octadecyl reversed-phase extraction columns (J.T. Baker, Deventer, The Netherlands), the crude hydroperoxides were analyzed by reversed-phase high-performance liquid chromatography on a CP-Spher-C18 column (5 μm, 4.6 × 250 mm) (Chrompack, Middelburg, The Netherlands) with tetrahydrofuran/methanol/water/acetic acid (25:30:45:0.1, by vol) at a flow rate of 1.0 mL min^{-1} as eluent. Detection was by a ultraviolet (UV) detector (Kratos Spectroflow 783, Separations, H.-I. Ambacht, The Netherlands) set at 234 nm. All preparative reactions were carried out at 25°C, by incubation of linoleic acid (500 μM) with either LOX-1 (36 nM) or LOX-3 (200 nM) in 250 mL air-saturated 0.1 M sodium borate (pH 9.0) or 0.1 M sodium phosphate buffer (pH 6.6), using reaction flasks with an airtight seal cap ($[O_2]$ = 240 μM). The appearance of secondary reaction products was monitored by the change in absorbance at 285 nm, corresponding to the absorption maximum of oxodienoic acids, in a Hewlett-Packard model 8450A UV-visible spectrophotometer (Hewlett-Packard, Amstelveen, The Netherlands).

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Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; LOX, lipoxygenase(s); TLC, thin-layer chromatography; UV, ultraviolet.

The reaction mixtures were acidified and extracted as described above. The free fatty acids were esterified by reaction with diazomethane and separated by preparative thin-layer chromatography (TLC) on precoated plates (Silicagel 60 F_{254}, 0.5 mm, E. Merck, Darmstadt, Germany) using the solvent system *n*-hexane/diethyl ether (40:60, vol/vol). The fractions, containing dimers were collected and, after extraction with methanol, hydrogenated over a platinum catalyst (PtO_2, MeOH). The hydrogenated fractions were analyzed on a Hewlett-Packard model 5890 series-II gas chromatograph equipped with a split injector. Gas chromatography (GC) was done under isothermal conditions for 2 min at 280°C followed by temperature programming to 320°C at 2°C min^{-1} using an HT-5 fused silica capillary column (25 m × 0.33 mm i.d., 1-μm phase thickness, Siloxane-Carborane, 5% phenyl equivalent; SGE, Ringwood Victoria, Australia) at a column pressure of 5 kPa with He as carrier gas (flow rate, 2 mL min^{-1}). The injector temperature was kept at 300°C. The column outlet was connected directly to the ionization source of a JEOL AX 505-W mass spectrometer (JEOL Ltd., Tokyo, Japan), operated at an ionization energy of 70 eV and kept at a temperature of 230°C. Gas chromatography/mass spectrometry (GC/MS) scanning was from *m/z* 35–700, and the following ions were selected in the mass chromatogram: *m/z* 297, 381, 409, 437, 463, 467, 491 and 594 (scan rate, 1 s^{-1}). The relative amounts of different positional isomers were determined by integration of the mass chromatograms.

RESULTS AND DISCUSSION

By use of a high-temperature capillary column (HT5) for the purification of the octadecadienoate dimers formed during the secondary reaction of soybean LOX, we were able to completely separate and analyze all positional isomers, as can be judged from the mass chromatograms obtained for the reaction of LOX-1 at pH 9.0 (Fig. 1). The mass spectra were collected at the center of each peak. Each fragmentation pattern is indicative of one type of linkage between the C_{18}-chains of the dimeric compounds only (Fig. 2 and Table 1). In addition to the major peaks, a small shoulder eluting at 10.7 min can be observed (Fig. 1, Trace 1). The mass chromatograms (Fig. 1) suggest that this shoulder could stem from a very minor amount of C(11)-C(11′)-linked positional isomer since the mass fragments characteristic for compounds branching at C(11) were more abundant, as compared to the spectra B [C(9)-C(11′)isomer] and D [C(11)-C(13′)-isomer] (Fig. 2). At least five positional isomers are formed during the secondary reaction of LOX-1 at pH 9.0 [C(9)-C(9′); C(9)-C(11′); C(9)-C(13′); C(11)-C(13′); C(13)-C(13′) and possibly a very minor amount of C(11)-C(11′)]. This finding extends the results reported by Garssen *et al.* (9), who detected only four of the positional isomers under identical reaction conditions and did not report any C(9)-C(9′)-linked compounds. The authors suggested that the formation of octadecadienoate dimers involves nonenzymic processes that can be conceived as a coupling of either two enzymically formed linoleic acid radicals or an enzymically formed linoleic acid radical and linoleate in the proximity of the enzyme (9). Studies on the reticulocyte enzyme corroborate the proposal of nonspecific association of linoleic acid radicals or a linoleic acid radical and linoleate (13).

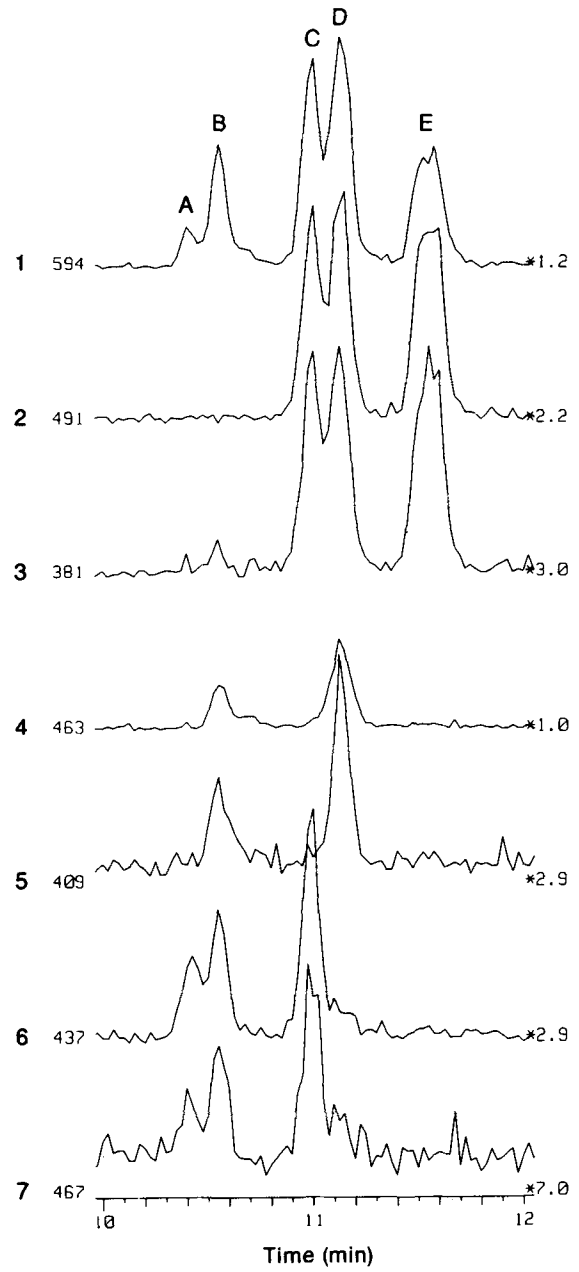

FIG. 1. Single-ion chromatograms from the gas chromatography/mass spectrometry analysis of octadecadienoate dimers from the reaction of lipoxygenase-1 with linoleic acid at pH 9.0. Trace 1: *m/z* 594, indicative of octadecadienoate dimers in general; Trace 2: *m/z* 491, indicative of C(13); Trace 3: *m/z* 381, indicative of C(13); Trace 4: *m/z* 463, indicative of C(11); Trace 5: *m/z* 409, indicative of C(11); Trace 6: *m/z* 437, indicative of C(9); Trace 7: *m/z* 467, indicative of C(9). A: C(9)-C(9′)-isomer; B: C(9)-C(11′)-isomer; C: C(9)-C(13′)-isomer; D: C(11)-C(13′)-isomer; E: C(13)-C(13′)-isomer.

Our results show that the relative amounts of isomeric octadecadienoate dimers formed during the secondary reactions of LOX-1 and LOX-3 (Fig. 3) remain unaltered, regardless of the regiospecificities found under aerobic conditions. This observation, and the fact that all positional isomers that could be expected from the reaction of free linoleic acid radicals in solution are formed, further corroborates the notion of nonenzyme controlled dimer formation. As only a very small amount of the

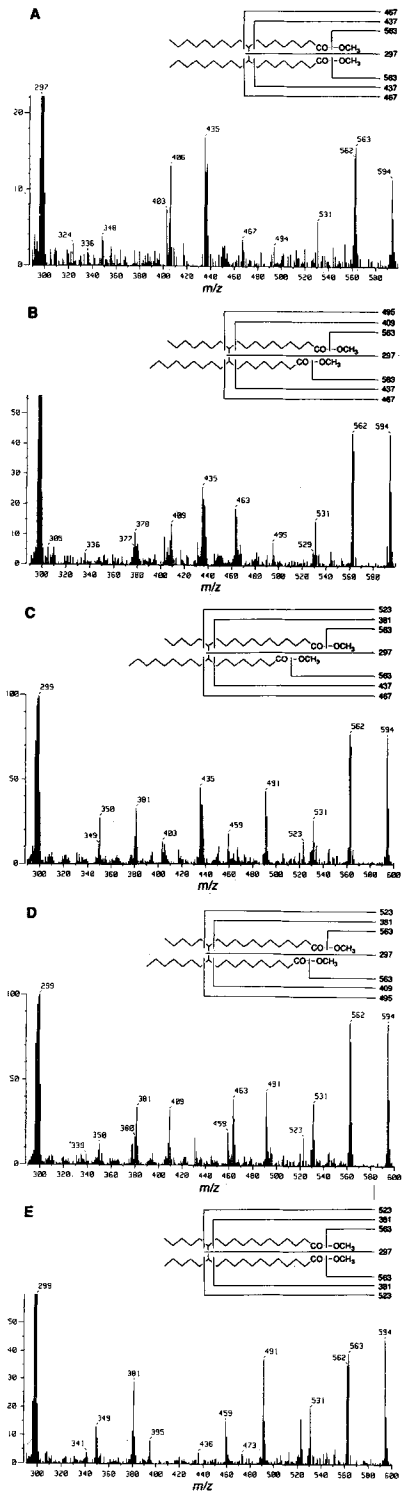

FIG. 2. Mass spectra of the regioisomeric octadecadienoate dimers from the secondary reaction of soybean lipoxygenase-1 with linoleic acid at pH 9.0. Lettering corresponds to that of the peaks in Figure 1 (Trace 1).

C(11)-C(11′)-linked isomer is produced, whereas the C(9)-C(11′) and the C(13)-C(11′)-linked isomers are among the major products, it can be concluded that the latter positional isomers are formed by the reaction of a free linoleic

TABLE 1

Characteristic Fragment Ions in the Mass Spectra of Esterified and Hydrogenated Octadecadienoate Dimers[a]

	m/z	*m/z* $-CH_3OH$	*m/z* $-2 \times CH_3OH$
$M^{+\bullet}$	594	562	
$[M - CH_3O]^+$	563	531	
$[M - C_5H_{11}]^{+a}$	523	491	459
$[M - C_7H_{15}]^{+b}$	495	463	431
$[M - C_9H_{19}]^{+c}$	467	435	403
$[M - C_7H_{14}CO_2CH_3]^{+c}$	437	405	
$[M - C_9H_{18}CO_2CH_3]^{+b}$	409	377	
$[M - C_{11}H_{22}CO_2CH_3]^{+a}$	381	349	
1/2 M^+	297	265	

[a]Mass fragments characteristic for: [a]C(13)-branching; [b]C(11)-branching; [c]C(9)-branching.

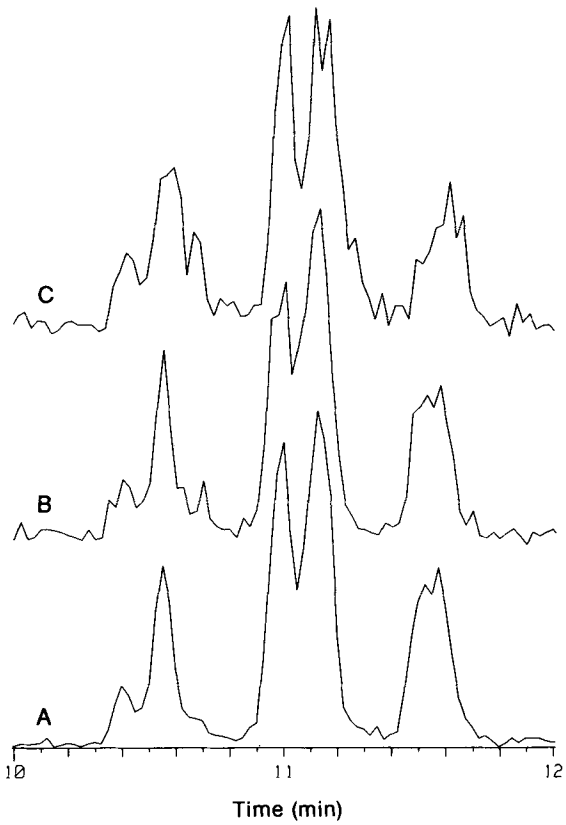

FIG. 3. Single-ion chromatograms (*m/z* 594) from the gas chromatography/mass spectrometry analysis of octadecadienoate dimers from the secondary reactions of lipoxygenases with linoleic acid under different conditions. Trace A: lipoxygenase-1/pH 9.0; Trace B: lipoxygenase-1/pH 6.6; Trace C: lipoxygenase-3/pH 6.6

acid radical with linoleate in solution. This is in agreement with the observation that only coupling to the C(13) and the C(9) positions of the linoleic acid radicals occurs in experiments in which the water-soluble radical scavenger 2-methyl-2-nitrosopropanol was used (14). Quantitative analyses of the octadecadienoate dimers show that in all cases dimeric compounds linked *via* C(13) are more abundant than those linked *via* C(9)-branched isomers (Table 2). Chan and Newby (15) have reported similar results for hemoprotein or metal-ion catalyzed peroxidations of linoleic acid in aqueous solution. These authors found that under a variety of reaction conditions13-hydroperoxylinoleic

TABLE 2

Percentage of Positional Isomer Branching Under Different Reaction Conditions[a]

	LOX-1/pH 9.0	LOX-1/pH 6.6	LOX-3/pH 6.6
C(9)-branching	36.3	37.3	38.8
C(13)-branching	63.7	62.7	61.2

[a]Values were obtained by integration of mass chromatograms. LOX, lipoxygenase.

acid constitutes 65–72%, and 9-hydroperoxylinoleic acid constitutes 28–35% of the total amount of hydroperoxy compounds formed in nonenzymic oxidations of linoleic acid. This result, and the observation that regioselectivity is greatly diminished when methyl linoleate was used as a substrate instead of the free acid, or when linoleic acid was oxidized in organic solvents, led them to suggest that the relative reactivity of C(9) and C(13) of linoleic acid in aqueous media is determined by the carboxylate group. The results reported here reflect a similarly remarkable deviation from the occurrence of C(13)- and C(9)-derived products in a 1:1 ratio.

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Divinyl Ethers and Hydroxy Fatty Acids from Three Species of *Laminaria* (Brown Algae)[1]

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Three species of brown algae, *Laminaria sinclairii, L. saccharina* and *L. setchellii*, have been investigated for the presence of oxylipins. From one, *L. sinclairii*, three new divinyl ether fatty acids have been characterized as methyl ester derivatives (methyl 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-6(*Z*), 9(*Z*),11(*E*)-dodecatrienoate, methyl 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoate, and methyl 14-[1′(*Z*),3′(*Z*)-hexadienyloxy]-5(*Z*),8(*Z*),11(*Z*),13(*E*)-tetradecatetraenoate) by a variety of spectroscopic methods. In addition, one new [13(*S*)-hydroxy-6(*Z*),9(*Z*),11(*E*),15(*Z*)-octadecatetraenoic acid] and four known monohydroxy polyunsaturated fatty acids have been isolated from all three species as their methyl ester derivatives. The occurrence of these compounds in brown algae strongly suggests that these organisms possess an active lipoxygenase(s) with ω6 specificity.
Lipids 28, 783–787 (1993).

Investigations of marine red algae have yielded a wide variety of oxylipins (1,2). Oxylipin has been introduced as an encompassing term for oxygenated compounds which are formed from fatty acids by reactions involving at least one step of mono- or dioxygenase-dependent oxidation (3,4). Representatives of these compounds in the brown algae have been scarce. The ecklonialactones (5,6), the epoxy and tetrahydrofuran lipids from *Notheia anomala* (7,8) and the cymathere ethers (9) are the only reported examples. As an extension of our work in this area of natural products chemistry, we examined several species of *Laminaria* and have discovered that these brown algae show evidence for active lipoxygenase metabolism (10).

MATERIALS AND METHODS

General. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (Karlsruhe, Germany) AC300 instrument operating at 300.13 MHz for ^{1}H NMR and at 75.46 MHz for ^{13}C NMR. Proton spectra were referenced to internal tetramethylsilane at 0.00 ppm. Carbon spectra were referenced to $CDCl_3$ at 77.00 ppm. Infrared (IR) spectra were obtained using a Nicolet (Madison, WI) 510 Fourier transform IR (FTIR) spectrometer. Ultraviolet (UV) spectra were run on a Hewlett-Packard (Palo Alto, CA) 8452a spectrophotometer. High-resolution electron-impact mass spectra (HR EIMS) were recorded on a Kratos (Manchester, England) MS 50 TC. High-performance liquid chromatography (HPLC) utilized a Waters (Milford, MA) M6000 pump, Rheodyne (Cotati, CA) 7125 injector and a Waters Lambda-Max 480 LC spectrophotometer. Optical rotations were obtained on a Perkin-Elmer (Norwalk, CT) 141 polarimeter. Merck (Darmstadt, Germany) aluminum-backed thin-layer chromatography (TLC) sheets (silica gel 60 F_{254}) were used for TLC. Compounds were detected by UV illumination or by heating plates sprayed with a 50% H_2SO_4 solution. Gas chromatography/mass spectrometry (GC/MS) was done utilizing a Hewlett-Packard 5890 Series II GC connected to a Hewlett-Packard 5971 mass spectrometer. Trimethylsilyl (TMS) ethers were prepared using TriSil® reagent (Pierce Chemicals, Rockford, IL). Hydrogenation reactions were carried out with 0.1–0.2 mg samples in ethanol using hydrogen gas and 5% palladium on activated carbon catalyst (Aldrich, Milwaukee, WI).

Collection, extraction and isolation. Laminaria sinclairii (Harvey *ex* Hooker f. *et* Harvey) Farlow, Anderson *et* Eaton was collected from the low intertidal at Strawberry Hill on the Oregon coast in May 1990. The algal material was immediately frozen with dry ice and stored at −20°C until extraction. Extraction of the defrosted alga (640 g dry wt) with warm $CHCl_3$/MeOH (2:1, vol/vol) provided 7 g of a viscous, dark brown oil which was fractionated by vacuum chromatography (7.5 cm × 5 cm, Merck Silica Gel G for TLC) using increasingly polar mixtures of hexanes and ethyl acetate. Fractions eluting with 40–50% EtOAc showed UV-active, brown charring reactions on TLC. These were combined (497 mg) and methylated with diazomethane. The methylated material was fractionated by flash chromatography (Merck Kieselgel 60, 230–400 mesh) using a stepped gradient from 5 to 20% EtOAc in hexanes. Fractions eluting with 5–10% EtOAc in hexanes were enriched with divinyl ether compounds, while those eluting with 20% EtOAc contained monohydroxy fatty acid methyl esters (236.6 mg). The divinyl ethers were further purified by HPLC (Maxsil Silica 10 μ, 500 mm × 10 mm; Phenomenex, Torrance, CA; 2.5% EtOAc/hexanes) to give ≈40 mg of methyl 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-6(*Z*), 9(*Z*),11(*E*)-dodecatrienoate (2) plus methyl 14-[1′(*Z*),3′(*Z*)-hexadienyloxy]-5(*Z*),8(*Z*),11(*Z*),13(*E*)-tetradecatetraenoate (4) and 3.4 mg of methyl 12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoate (6). Compounds 2 and 4 were unstable, and attempts to separate these compounds led to their degradation. The monohydroxy compounds (50.7 mg portion) were fractionated initially by normal-phase HPLC (Maxsil Silica 10 μ, 500 mm × 10 mm, 15% EtOAc/hexanes) and then by reverse-phase HPLC (Merck Lichrosorb RP-18 7 μ, 250 mm × 10 mm, 85% MeOH/H_2O) to yield: (i) a mixture of methyl 13(*S*)-hydroxy-6(*Z*),9(*Z*),11(*E*),15(*Z*)-octadecatetraenoate (8) and minor related species (ii) methyl 15(*S*)-hydroxy-5(*Z*),8(*Z*), 11(*Z*),13(*E*),17(*Z*)-eicosapentaenoate (10), methyl 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoate (12), and methyl 12-hydroxy-9,13,15-octadecatrienoate (17) and (iii) methyl 15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate (14) plus methyl 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate

[1]A preliminary summary of this work was presented at the XIVth International Seaweed Symposium, Brest, France, August 1992 (10).
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Abbreviations: COSY, chemical shift correlation spectroscopy; FTIR, Fourier transform infrared; GC/MS, gas chromatography/mass spectrometry; 15-HEPE, 15-hydroxyeicosapentaenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; 13-HODTA, 13-hydroxyoctadecatetraenoic acid; 13-HOTE, 13-hydroxyoctadecatrienoic acid; HPLC, high-performance liquid chromatography; HR EIMS, high-resolution electron-impact mass spectrometry; IR, infrared; LR EIMS, low-resolution electron-impact mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl; UV, ultraviolet; XHCORR, heteronuclear chemical shift correlation spectroscopy.

(16). Final purification of compounds 8 (5.9 mg), 14 (4.7 mg), and 16 (2.4 mg) was accomplished by HPLC (Versapack Silica 10 μ, 2 × 4.1 mm × 30 cm; Alltech, Deerfield, IL; 10% EtOAc/hexanes). Separation of compound 10 (6.4 mg) and a 4:1 mixture of 12 and 17 (4.8 mg; ratio determined by integration of GC peaks) was also achieved by HPLC (Versapack Silica 10 μ, 2 × 4.1 mm × 30 cm, 0.75% isopropanol/hexanes). All pure compounds were colorless oils.

Laminaria setchellii Silva was collected at Boiler Bay on the Oregon coast in April, 1991, and *L. saccharina* (L.) Lamouroux was obtained from Puget Sound, Washington in July 1990. Both of these algae were processed in a manner similar to the above procedure to obtain compounds 8, 10, 12, 14 and 16. Neither of these algae showed any evidence for the divinyl ether fatty acids.

Methyl 12-[1′(Z),3′(Z)-hexadienyloxy]-6(Z),9(Z),11(E)-dodecatrienoate (2). A colorless oil. UV (MeOH) λ_{max} = 268 nm (ε = 30000); FTIR (neat) 3013, 2963, 2934, 1739, 1646, 1595, 1434, 1261, 1228, 1163 cm^{-1}. For ^{1}H and ^{13}C NMR data, see Table 1. Low-resolution electron-impact mass spectrum (LR EIMS) *m/z* (rel. intensity) 304 (M^+, 12), 275 (4), 175 (10), 105 (38), 91 (82), 79 (100). HR EIMS: Found, 304.204; calcd. for $C_{19}H_{28}O_3$ (M^+), 304.204. LR EIMS *m/z* (rel. intensity) of hydrogenated 2: 283 (M^+-OCH_3, 3), 229 (47), 214 (36), 197 (100), 181 (20), 171 (27), 163 (24), 143 (60), 129 (22), 97 (46), 87 (87), 74 (85), 69 (51), 55 (63).

Methyl 14-[1′(Z),3′(Z)-hexadienyloxyl]-5(Z),8(Z),11(Z),13(E)-tetradecatetraenoate (4). LR EIMS *m/z* (rel. intensity) of 4 or a geometrical isomer: 330 (M^+, 1), 250 (10), 233(8), 201 (20), 183 (33), 159 (16), 150 (23), 131 (27), 121 (100), 117 (46), 105 (40), 91 (85), 79 (77), 67 (51). LR EIMS *m/z* (rel. intensity) of hydrogenated 4: 342 (M^+, 0.5), 311 (M^+-OCH_3, 2), 257 (29), 242 (31), 225 (42), 199 (23), 143 (44), 111 (25), 97 (44), 87 (83), 74 (100), 69 (59), 55 (62).

Methyl 12-[1′(Z),3′(Z)-hexadienyloxy]-9(Z),11(E)-dodecadienoate (6). A colorless oil. UV (EtOH) λ_{max} = 268 nm (ε = 30000); FTIR (neat) 2929, 2855, 1740, 1647, 1595, 1434, 1250, 1228, 1164 cm^{-1}. LR EIMS *m/z* (rel. intensity) 306 (M^+, 49), 275 (5), 245 (6), 149 (35), 131 (40), 107 (34), 96 (48), 81 (100), 67 (86), 55 (96). For ^{1}H NMR data, see Table 1. HR EIMS: Found, 306.220; calcd. for $C_{19}H_{30}O_3$ (M^+), 306.219.

Methyl 13(S)-hydroxy-6(Z),9(Z),11(E),15(Z)-octadecatetraenoate (8). A colorless oil. $[\alpha]_D^{27}$ = +12° (c = 0.66, acetone), UV (MeOH) λ_{max} = 238 nm (ε = 27000); FTIR (neat) 3438 (broad), 3011, 2960, 2934, 1740, 1437, 1212, 1202, 1175, 985 cm^{-1}. LR EIMS *m/z* (rel. intensity) 306 (M^+, 0.5), 288 (M^+-H_2O, 7), 275 (2), 237 (35), 219 (22), 205 (41), 187 (40), 159 (50), 107 (78), 81 (100), 69 (57). For ^{1}H and ^{13}C NMR data, see Table 1. HR EIMS: Found, 306.219; calcd. for $C_{19}H_{30}O_3$ (M^+), 306.219. LR EIMS *m/z* (rel. intensity) of TMS derivative: 363 (M^+-CH_3, 0.7), 347 (M^+-OCH_3, 0.7), 309 (100), 243 (10), 219 (6), 187 (10), 145 (19), 129 (16), 103 (21), 91 (24), 73 (89).

Methyl 15(S)-hydroxy-5(Z),8(Z),11(Z),13(E),17(Z)-eicosapentaenoate (10). A colorless oil. $[\alpha]_D^{29.5}$ = +9.9° (c = 0.71, acetone). Based on the known positive optical rotation for ethyl 15-*S*-HETE (11), this positive rotation also indicates *S* stereochemistry at position-15. The predominantly *S* stereochemistry was confirmed by oxidative ozonolysis of the (−)-menthoxycarbonyl derivative (12) and subsequent comparison by GC/MS of the malate derivative with standards prepared from D- and L-malate (13). The experimentally obtained malate fragment significantly enriched the peak corresponding to the L-malate derivative when co-injected into the GC/MS with a mixture of D- and L-standards (due to incomplete resolution of the D- and L-malate-derived standards under our GC/MS conditions, quantitative determination of the *R* and *S* enantiomers was not possible). ^{1}H

TABLE 1

NMR Data for the Methyl Ester Derivatives of Oxylipins from *Laminaria sinclairii*[a]

	Methyl divinyl ether 2				Methyl divinyl ether 6				Methyl 13-HODTA (8)			
	^{1}H			^{13}C[b]	^{1}H				^{1}H			^{13}C[b]
C No.	ppm	m	J(Hz)	ppm	ppm	m	J(Hz)		ppm	m	J(Hz)	ppm
1	—	—	—	174.12	—	—	—		—	—	—	174.22
2	2.32	*t*	7.5	33.93	2.30	*t*	7.5		2.32	*t*	7.6	33.93
3	1.65	*tt*	7.8, 7.5	24.53	1.62	*bt*	7.3		1.65	*tt*	7.6, 7.6	24.52
4	1.40	*tt*	7.8, 7.4	29.03	1.4-1.2	*m*	—		1.39	*tt*	7.6, 7.6	28.99
5	2.09	*bdt*	7.5, 7.4	26.83	1.4-1.2	*m*	—		2.08	*m*	—	26.87
6	5.38	*m*	—	129.83	1.4-1.2	*m*	—		5.38	*m*	—	129.99
7	5.38	*m*	—	127.83	1.4-1.2	*m*	—		5.38	*m*	—	127.61
8	2.86	*bdd*	7.5, 7.2	26.00	2.10	*m*	—		2.93	*bddd*	6.2, 6.8, 1.5	26.12
9	5.26	*dt*	10.5, 7.5	127.30	5.30	*dt*	10.8, 7.6		5.38	*m*	—	130.41
10	5.86	*dbb*	11.1, 10.5	123.32	5.85	*bdd*	11.3, 10.8		5.99	*bdd*	11.1, 10.9	127.81
11	6.07	*dd*	11.9, 11.1	106.48	6.05	*bdd*	11.9, 11.3		6.56	*ddt*	15.2, 11.1, 1.2	125.39
12	6.61	*d*	11.9	147.91	6.59	*d*	11.9		5.73	*dd*	15.2, 6.3	135.73
13	6.21	*d*	6.2	141.79	6.20	*d*	6.2		4.23	*m*	—	72.02
14	5.51	*ddd*	11.5, 6.2, 0.9	105.70	5.50	*ddd*	11.5, 6.2, 1.2		2.33	*m*	—	35.23
15	6.32	*bdd*	11.5, 11.3	119.54	6.32	*bdd*	11.5, 11.1		5.38	*m*	—	123.76
16	5.43	*m*	—	133.09	5.42	*bdt*	11.1, 7.5		5.57	*dtt*	10.2, 7.2, 1.4	135.18
17	2.16	*dqd*	7.5, 7.5, 1.4	20.98	2.16	*dqd*	7.5, 7.5, 1.5		2.08	*m*	—	20.73
18	1.00	*t*	7.5	14.11	1.00	*t*	7.5		0.97	*t*	7.5	14.19
OMe	3.67	*s*	—	51.46	3.67	*s*	—		3.67	*s*	—	51.51
OH	—	—	—	—	—	—	—		1.91	*d*	3.9	—

[a]^{1}H chemical shifts are reported relative to internal tetramethylsilane at 0.00 pm. ^{13}C shifts are referenced to $CDCl_3$ at 77.00 ppm. All spectra were recorded in $CDCl_3$. NMR, nuclear magnetic resonance; 13-HODTA, 13-hydroxyoctadecatetraenoic acid.
[b]Assignments based on a ^{1}H-^{13}H-^{13}C heteronuclear chemical shift correlation spectroscopy experiment and by comparison to model compounds (15).

NMR ($CDCl_3$; δ) 6.56 (1H, *dddd*, J = 15.2, 11.1, 1.1, 1.1 Hz, H-13), 6.00 (1H, *dd*, J = 11.1, 10.8 Hz, H-12), 5.73 (1H, *dd*, J = 15.2, 6.3 Hz, H-14), 5.58 (1H, *bdt*, J = 10.8, 7.2 Hz, H-18), 5.39 (6H, *m*, H-5, H-6, H-8, H-9, H-11, H-17), 4.23 (1H, *m*, H-15), 3.67 (3H, *s*, OCH_3), 2.96 (2H, *bdd*, J = 6.3, 6.0 Hz, H-10), 2.81 (2H, *bdd*, J = 5.4, 5.3 Hz, H-7), 2.34 (2H, *m*, H-16), 2.33 (2H, *t*, J = 7.4 Hz, H-2), 2.10 (2H, *m*, H-4), 2.09 (2H, *m*, H-19), 1.84 (1H, *d*, J = 4.0 Hz, OH), 1.71 (2H, *tt*, J = 7.4, 7.4 Hz, H-3), 0.97 (3H, *t*, J = 7.5 Hz, H-20). ^{13}C NMR [$CDCl_3$; assignments based on model compounds (compound 8 and Ref. 11); δ]: 174.14 (C-1), 135.77 (C-14), 135.27 (C-18), 130.21 (C-11), 128.74 (C-5), 128.98 (C-6), 128.59 (C-8), 127.97 (C-9), 127.56 (C-12), 125.34 (C-13), 123.70 (C-17), 71.98 (C-15), 51.53 (OCH_3), 35.26 (C-16), 33.40 (C-2), 26.52 (C-4), 26.10 (C-10), 25.62 (C-7), 24.72 (C-3), 20.73 (C-19), 14.20 (C-20).

Methyl 13(S)-hydroxy-9(Z),11(E),15(Z)-octadecatrienoate (**12**). A colorless oil. $[\alpha]_D^{29} = +5.1°$ (c = 0.44, acetone) for a 4:1 mixture (by GC integration) of **12** and **17**.

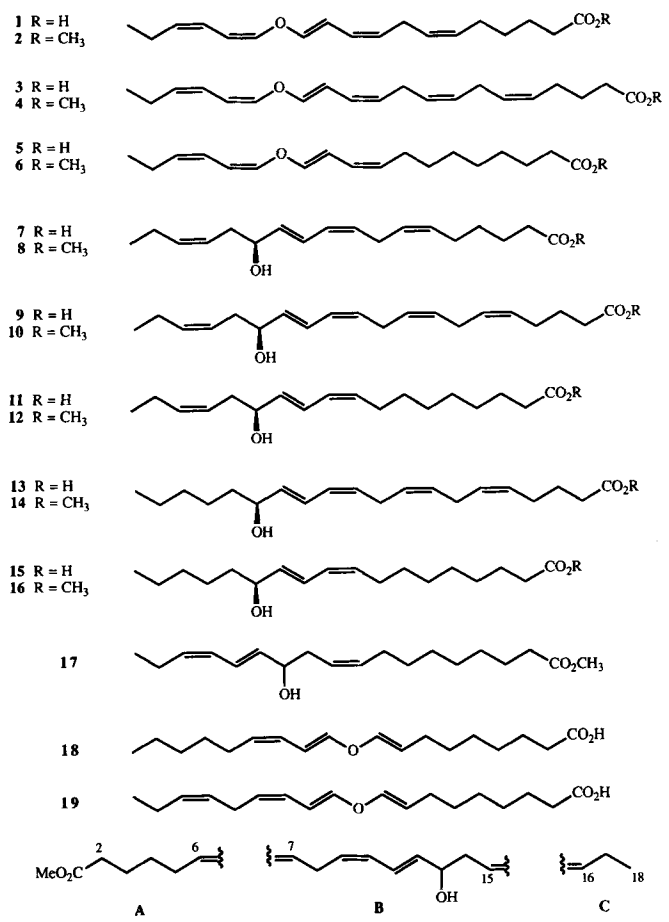
Methyl 15(S)-hydroxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoate (**14**). A colorless oil. $[\alpha]_D^{32} = +9.0°$ (c = 0.52, acetone), $[\alpha]_D^{32} = +4.4°$ (c = 0.52, hexanes), $[\alpha]_D^{32} = +8.8°$ (c = 0.52, EtOH). ^1H and ^{13}C NMR data correlated well with previously reported values (11).

Methyl 13(S)-hydroxy-9(Z),11(E)-octadecadienoate (**16**). A colorless oil. $[\alpha]_D^{27} = +8.9°$ (c = 0.27, acetone).

Methyl 12-hydroxy-9,13,15-octadecatrienoate (**17**). [This compound was previously characterized from a green alga (14).] LR EIMS *m/z* (rel. intensity) 308 (M^+, 0.15), 290 (M^+-H_2O, 3), 227 (2), 195 (3), 133 (3), 111 (100), 93 (12), 81 (11), 67 (12), 55 (14). A C_{18} hydroxy acid methyl ester with three double bonds was indicated by the ion at *m/z* 308 (M^+). The base peak at *m/z* 111 suggested placement of the hydroxy at C-12 with a diene unit oriented toward the methyl terminus. Assuming an α-linolenic acid precursor to this compound, the third olefin was positioned at C-9. The LR EIMS spectrum of the TMS-derivative of the hydrogenated species matched well with an authentic spectrum of methyl 12-(TMS)oxyoctadecanoate (91% match with spectrum #45118 of the National Bureau of Standards mass spectral library #NBS54k included with the Hewlett-Packard 5971 MS operating software), confirming the placement of the hydroxyl at position-12. The stereochemistries of the olefins for compound **17** are proposed based on biogenetic arguments.

RESULTS AND DISCUSSION

Laminaria sinclairii was collected on the Oregon coast and immediately frozen with dry ice. Extraction of the defrosted alga with warm $CHCl_3$/MeOH provided a viscous, dark brown oil which was fractionated by vacuum silica gel chromatography. Several nonpolar fractions showed UV active, brown-charring spots on TLC. These were combined, methylated with CH_2N_2, and further fractionated to provide material rich in divinyl ethers and monohydroxy fatty acid methyl esters. The divinyl ethers were finally purified by normal-phase HPLC to yield a 4:1 mixture of compounds **2** and **4** (determined by GC integration of hydrogenated **2** and **4**) and pure compound **6**. Attempts to isolate **2** and **4** in pure form were unsuccessful as the compounds degraded with each step of purification.



Since compounds **2** and **4** were not separable without extensive decomposition, data were collected for the mixture. The ^1H NMR spectrum showed minor shoulders on several of the olefinic peaks and distortions in upfield patterns indicating that **2** and **4** were closely related. The ^{13}C NMR data (Table 1) and HR EIMS provided a molecular formula of $C_{19}H_{28}O_3$ for the major component. An analysis of the NMR data revealed five olefins and one ester carbonyl. Despite the presence of compound **4**, two isolated spin systems [H(2)-H(12) and H(13)-H(18)] were obtained from the ^1H-^1H chemical shift correlation spectroscopy (COSY) spectrum. Since two of the oxygens in the formula are contained in the ester function, the remaining oxygen logically would connect the two spin systems. The resulting divinyl ether structure explains the polarization of the olefinic carbons at the termini of these spin systems (C-12, δ147.91, C-11, δ106.48; C-13, δ141.79, C-14, δ105.70). The magnitudes of the coupling constants for the olefins adjacent to the ether oxygen are also consistent with a divinyl ether structure. Enol ethers show distinctive coupling constants with J_{trans} = 12.0-12.6 Hz and J_{cis} = 6.2-6.7 Hz (15). Thus, the C(11)- C(12) double bond was *trans* (11.9 Hz) while the C(13)-C(14) olefin was *cis* (6.2 Hz). The C(9)-C(10) (10.5 Hz) and C(15)-C(16) (11.3 Hz) protons showed coupling constants of more typical *cis* olefins. The C(5)-C(6) olefin was also *cis* based on the ^{13}C shifts of the adjacent methylene carbons (16) (H-6 and H-7 were a degenerate multiplet). Hence, structure **2** was defined as methy 12-[1′(Z),3′(Z)-hexadienyloxy]-6(Z),9(Z),11(E)-dodecatrienoate.

The ^1H-^1H COSY spectrum of the mixture of 2 and 4 suggested that compound 4 was a C_{20} analog based on an extra correlation from δ2.10 (H-4) to δ1.71 (H-3). The H-3 protons of Δ5 C_{20} polyunsaturated fatty acids and derivatives typically occur at δ1.71. Also, the bisallylic methylene at δ2.86 in 2 appeared to have small, broad, doublets of doublets obscured to both the downfield and upfield sides of the main pattern, indicating the presence of two bisallylic methylenes in compound 4. Analysis by GC/MS supports this conclusion. Although divinyl ethers 2 and 4 thermally isomerize to olefin isomers under GC/MS conditions, a peak that eluted after the major enol ether 2 showed a peak at *m/z* 330 which corresponds to the M^+ for a C_{20} species with six olefins. A fragment peak at *m/z* 233 indicated an ether oxygen between C-14 and C-15. The mixture of divinyl ethers 2 and 4 was hydrogenated, and the derivatized minor component had a M^+ 342 peak which implied six olefins in 4 and a major fragment peak at *m/z* 257, consistent with the loss of a C_6H_{13} unit to the distal side of the oxygen. Since the ^1H NMR patterns of 2 and 4 essentially overlap and the above GC/MS evidence supports a C_{20} analog with six double bonds, we propose structure 4, methyl 14-[1′(*Z*), 3′(*Z*)-hexadienyloxy]-5(*Z*),8(*Z*),11(*Z*),13(*E*)-tetradecatetraenoate, for the minor component. The olefin geometries shown are consistent with the functionality of 2 and the likely C_{20} fatty acid precursor, 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid.

Compound 6, the least polar divinyl ether from *L. sinclairii*, contained the same chromophore as in 2 (λ_{max} 268 nm). The ^1H NMR spectrum of 6 was highly comparable to that of 2, except for two less olefinic protons and four more protons in the high field region of the spectrum (see Table 1). This suggested that 6 was the C(6)-C(7) dihydro analog of 2 which was further supported by ^1H-^1H COSY analysis. Confirmation of this structure was obtained from mass spectral analysis. A significant M^+ 306 peak was present (49%), and the base peak at *m/z* 81 is consistent with cleavage to the distal side of the ether oxygen. Hydrogenation of compounds 2 and 6 and subsequent GC/MS analysis showed that these two hydrogenated products were identical.

The only known examples of divinyl ether fatty acids in nature are colneleic (18) and colnelenic (19) acids from potato tubers (17) and polyneuric acid from the marine red alga *Polyneura latissima* (18). Compounds 1 and 5 differ from colneleic and colnelenic acids in the position of oxygenation and in the double-bond stereochemistry about the oxygen linkage. Colneleic and colnelenic acids have a *trans* olefin on either side of the ether oxygen, while compounds 1 and 5 have a *cis* double bond to one side of the oxygen and a *trans* olefin on the other side. The potato tuber fatty acids arise from a pathway initiated by a 9-lipoxygenase acting on linoleic or α-linolenic acid (19). Assuming an analogous pathway to the new compounds, a 13-lipoxygenase is indicated in the formation of 1 and 5 from C_{18} precursors and a 15-lipoxygenase in the formation of 3 from a C_{20} precursor (ω6 in each case). Divinyl ether 1 would derive from stearidonic acid, and compound 5 would arise from α-linolenic acid.

The complex mixture of monohydroxy polyunsaturated fatty acid methyl esters from *L. sinclairii* was purified by a combination of normal- and reverse-phase HPLC to yield five major components: 8, 10, 12, 14 and 16. Four of these were known compounds: methyl 15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*),17(*Z*)-eicosapentaenoate [methyl 15(*S*)-HEPE, 10], methyl 13(*S*)-hydroxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoate [methyl 13(*S*)-HOTE, 12], methyl 15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoate [methyl 15(*S*)-HETE, 14] and methyl 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoate [methyl 13(*S*)-HODE, 16]. Methyl 15(*S*)-HETE, methyl 15(*S*)-HEPE and methyl 13(*S*)-HOTE were identified by ^1H NMR, ^{13}C NMR, ^1H-^1H COSY, optical rotations and GC/MS analysis of TMS derivatives and comparison to literature data (11,20–24). Methyl 13(*S*)-HODE was characterized by ^1H NMR, optical rotation (25) and GC/MS analysis of the TMS derivative (26). The fifth compound, 8, was closely related to the above hydroxy acid methyl esters by NMR and analyzed for $C_{18}H_{30}O_3$ by HR EIMS. The H(2)–H(6) (**A**), H(7)–H(15) (**B**) and H(16)–H(18) (**C**) spin systems could be assembled based on ^1H-^1H COSY data, but due to overlap in the olefinic and allylic methylene regions, connection of these fragments was not possible. The two possibilities for combining these fragments gave rise to either a 9-hydroxy or a 13-hydroxy compound. Biogenetic arguments would favor the 13-hydroxy compound based on the co-occurrence of methyl 13-HOTE and methyl 13-HODE. LR EIMS supported this by showing a significant fragment at *m/z* 237, corresponding to cleavage distal to C-13. Further proof for oxidation at C-13 in 8 was obtained by separately hydrogenating small amounts of methyl 13-HODE, methyl 13-HOTE and compound 8 and analyzing the products as TMS ethers by GC/MS. All three samples were analyzed for the TMS ether of methyl 13-hydroxystearate (27). Carbon NMR shifts in 8 (Table 1) were assigned based on a ^1H-^{13}C XHCORR experiment and model compounds (11). The positive optical rotation obtained for this sample indicated *S* stereochemistry at position-13, based on the known positive rotations of methyl 13(*S*)-HOTE (24) and methyl 13(*S*)-HODE (25). This stereochemical assignment was verified by analyzing the ozonolysis product of the (−)-menthoxycarbonyl derivative (12) by GC/MS and comparison to known standards (13). The malate-derived product significantly enriched the peak corresponding to the L-malate derivative when co-injected into the GC/MS with a mixtue of D- and L-standards. These data allowed the assignment of the new structure as methyl 13(*S*)-hydroxy-6(*Z*),9(*Z*),11(*E*),15(*Z*)-octadecatetraenoate (methyl 13(*S*)-HODTA, 8). All five of these hydroxy acid derivatives were also obtained in 0.15–0.4% yield from extracts of *L. setchellii* and *L. saccharina* (10).

The occurrence of these hydroxy acids 7, 9, 11, 13, and 15 in several species of *Laminaria* suggests that algae of this genus possess a lipoxygenase with positional specificity for C-13 in C_{18} substrates and C-15 in C_{20} substrates (ω6 in both structure classes). The presence of oxylipins in *Ecklonia stolonifera* (5,6), *Cymathere triplicata* (9) and now in several species of *Laminaria* suggests that other brown algae of the Laminariaceae offer a potential source of these novel fatty acid derivatives. The physiological relevance of these compounds in the algae is unknown, but related compounds appear to act as endogenous elicitors of phytoalexin production in rice plants which have been challenged with a fungal infection (28). The role that the divinyl ethers play is also unknown, although colneleic acid has shown *in vitro* activity as a 5-lipoxygenase inhibitor (29).

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Age-Dependent Accumulation of Phosphatidylcholine Hydroperoxide in the Brain and Liver of the Rat

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Age-related changes in phosphatidylcholine hydroperoxide (PCOOH) content as an index for oxidative membrane lipid damage were determined by high-performance liquid chromatography using chemiluminescence detection. Brain and liver PCOOH content increased significantly in male and female rats with age. The brain PCOOH content of male 18-month-old rats was 4.4 times that of 1-month-old rats, and that of female 18-month-old rats was 3.5 times that of 1-month-old females. The liver PCOOH content of the male 18-month-old rats was 9.3 times that of the 1-month-old; and of the female 18-month-old rats was 4.7 times that of the 1-month-old. PCOOH levels in heart and lung did not show age dependency. In both brain and liver (but not in heart and lung), the phosphatidylcholine content significantly decreased upon aging. The results indicate that oxidative deterioration, such as phospholipid hydroperoxidation, is prevalent in the membrane lipids of brain and liver of the rat due to aging.
***Lipids* 28, 789–793 (1993).**

Peroxidation of membrane lipids has been implicated as one of the basic mechanisms of age-related pathological changes (1–3). The thiobarbituric acid (TBA) assay has previously been employed to estimate lipid peroxides in human plasma (4) and in tissues of rodents as a function of age (5–11). Several investigators have reported an increase in TBA reactants in rat brain (5,8,9,11) and liver (8,9,11) with aging whereas others could not show such changes (6,7,10). These inconsistencies may well be attributable to methodological problems, because the TBA assay also responds to aldehydes, amino acids and carbohydrates unrelated to lipid peroxides (12,13).

Alternatively, expiration of hydrocarbon gas by aged rats (14), conjugated diene absorption of tissue lipids upon aging (15) and age-related fluorescence pigment formation (16) have been measured to follow membrane lipid hydroperoxide formation. However, to accurately assess the degree of lipid hydroperoxidation in biological membranes, the direct measurement of primary peroxidation products, such as phospholipid hydroperoxides, is most desirable.

A new, sensitive and selective assay of phospholipid hydroperoxides by high-performance liquid chromatography (HPLC) combined with chemiluminescence (CL) detection was first described by Miyazawa and colleagues (17–20). This CL–HPLC method was successfully applied to measure phospholipid hydroperoxides present in human plasma (19, 21), serum lipoproteins (22) and in liver and brain of rodents (20,23,24). More recently, this method was used to establish that the phosphatidylcholine hydroperoxide (PCOOH) content of *Drosophila melanogaster* flies is reciprocally proportional to the flies' life span (25,26). PCOOH accumulation in cultured human diploid cells, upon reaching cellular population doubling levels, was also measured by this method (27). A similar HPLC method with CL detection was independently described by Frei *et al.* (28), although these authors could not detect phospholipid hydroperoxides in human blood plasma due to methodological limitations.

In the present paper, we primarily determined the age-related changes that occur in PCOOH levels in the internal organs of the rat using the CL–HPLC method to follow the oxidative damage of membrane lipids in this *in vivo* rodent system.

MATERIALS AND METHODS

Animals. Male and female Sprague-Dawley rats (n = 42) were fed a standard laboratory diet (F-2 pellet rations; Funabashi Farm Co., Chiba, Japan; containing 5 mg of vitamin E per 100 g of diet) *ad libitum* for 1, 7, 12 and 18 mon for male rats (body weight, 140, 557, 678 and 722 g, respectively; n = 6 for each group); and for 1, 7 and 12 mon for female rats (body weight, 112, 334 and 443 g, respectively; n = 6 for each group). Groups of three rats each were housed in stainless steel cages and maintained at 25°C on a 12 h (8 a.m. to 8 p.m.) light–dark cycle. The rats were fasted for 20 h prior to dissection and were sacrificed by exsanguination under light diethyl ether anesthesia. The livers were perfused *in situ* with ice-cold 0.15 M saline, and then the brain, liver, heart and lung were removed.

Total lipid (TL) extraction. TL were extracted with a mixture of chloroform and methanol (2:1, vol/vol) (29) from brain, liver, heart and lung as described previously (23,24).

CL–HPLC. The CL–HPLC system and conditions for measuring PCOOH in total lipids from rat tissues were essentially the same as those previously described by Miyazawa *et al.* (23,24).

Determination of phosphatidylcholine (PC) and α-tocopherol. The PC content in tissue TL was measured by an Iatroscan thin-layer chromatography (TLC)/flame-ionization detection method using an Iatroscan TH-10 apparatus (Iatron Laboratories Inc., Tokyo, Japan) as described by Hazel (30). α-Tocopherol content was measured by the fluorescence-HPLC method (31).

Fatty acid analysis. PC was separated from tissue TL lipids by TLC (silica gel 60; Merck, Darmstadt, Germany) using chloroform/methanol/acetic acid/water (25:15:4:2, by vol) as developing solvent (32). Fatty acid methyl esters derived from TL and from PC were prepared by acid-catalyzed transmethylation (33). Methyl esters were extracted with hexane and were analyzed using a Shimadzu GC-8A gas chromatograph equipped with a glass column (300 × 0.2 cm) containing 10% Silar 10C on Chromosorb W (60/80 mesh; Supelco, Bellefonte, PA). Column temperature was programmed from 170 to 220°C at 1°C/min. Fatty acid methyl esters were identified by comparison of their retention times with those of standards.

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Abbreviations: CL, chemiluminescence; DHA, docosahexaenoic acid; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; TBA, thiobarbituric acid; TL, total lipids; TLC, thin-layer chromatography.

Statistical analysis. The data are expressed as mean values with standard deviations. All data were analyzed using the Student's *t*-test.

RESULTS

Figure 1 shows the CL chromatograms of PCOOH from brain TL of young male rats (1-month-old, Fig. 1A) and of aged male rats (18-month-old, Fig. 1B). PCOOH (retention time at 11.0 min) was clearly separated and detected as a single sharp peak by CL detection. This peak had the same retention time as did authentic PCOOH; its identity was further confirmed by cochromatography with authentic PCOOH. The chemiluminescent peak due to the hydroperoxide group in PCOOH completely disappeared upon reduction of the tissue TL with sodium borohydride to give the corresponding hydroxyl derivative, as has been previously reported (18–20). As shown in Figure 1, the PCOOH peak intensity observed for the brain sample from 18-month-old rats (767 pmol PCOOH/g brain) was more intense than that for 1-month-old rats (140 pmol PCOOH/g brain). By measuring conjugated diene absorption (lower chart of Fig. 1), a slight, but insignificant, increase was observed for 18-month-old rats compared to 1-month-old rats. The lack of significance was probably due to the comparatively low sensitivity of the conjugated diene method.

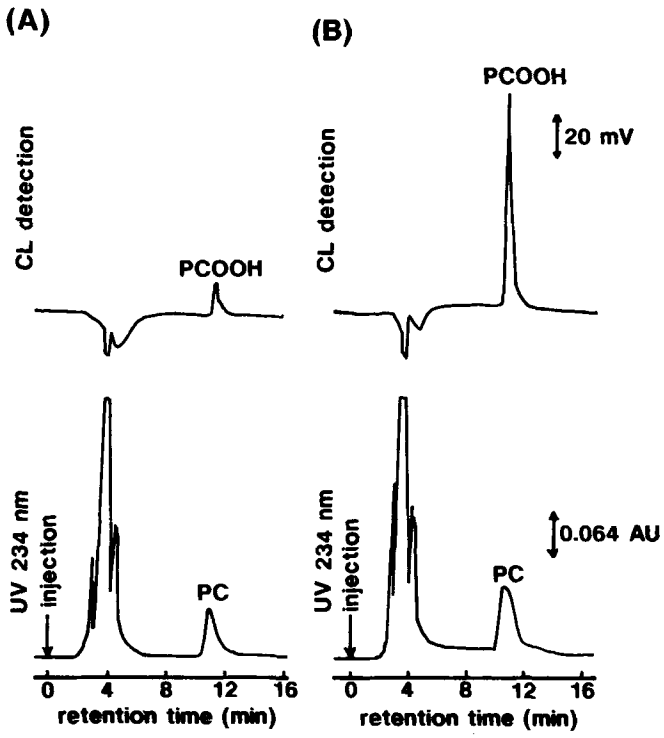

FIG. 1. Chemiluminescence (CL)-high-performance liquid chromatography chromatograms of phosphatidylcholine hydroperoxides (PCOOH) from brain total lipids prepared from male rats. Panel A, 1-month-old rats. Panel B, 18-month-old rats. The PCOOH concentration detected on the chemiluminescent chromatogram corresponded to 140 pmol/g brain (A) and 767 pmol/g brain (B). The upper chart shows CL detection, the lower chart shows conjugated diene (234 nm) detection. UV, ultraviolet.

Brain. For both sexes, brain weights, TL and α-tocopherol content at 7, 12 and 18 mon were significantly higher than those at one month (Table 1). The brain PC content expressed per brain weight was constant during the 18-mon feeding period, but the PC content in the TL showed an age-dependent decrease for both sexes. The brain PCOOH was increased with aging in both male and female rats. The brain PCOOH content of the 18-month-old male rats was 4.4 times that of the 1-month-old, and of the 18-month-old female rats was 3.5 times higher than that of the 1-month-old.

Liver. Liver weights increased with aging (Table 2). However, TL contents were not age-dependent, except for the 18-month-old female rats that had the highest content. The PC content of TL in males was lower at 12 and 18 mon than at 1 and 7 mon, and in females PC content was lower at 18 mon than at 1 and 7 mon. α-Tocopherol contents both in male and female rats were not age-dependent. Female liver α-tocopherol contents were significantly higher than those of male livers for all age groups examined. Liver PCOOH increased proportionally with age in both sexes. The PCOOH content of the 18-month-old male rats was 9.3 times higher than that of the 1-month-old, and that of the 18-month-old female rats was 4.7 times that of the 1-month-old. Female rats had a higher liver PCOOH content at the early ages (1- and 7-month-old) compared with male rats, and the 18-month-old females had a lower liver PCOOH content than the 18-month-old males.

Heart and lung. The heart weights of the 7-, 12- and 18-month old rats were higher than those of the 1-month-old rats (Table 3). Lung weights increased with aging. TL

TABLE 1

Changes in Lipid Composition and in α-Tocopherol and Phosphatidylcholine Hydroperoxide (PCOOH) Contents of Rat Brain with Aging[a]

Rats (mon)	Brain weight (g)	Total lipids (TL) (mg/g brain)	PC (mg/g brain)	PC (mg/g TL)	α-Tocopherol (μg/g brain)	PCOOH (pmol/g brain)
Male						
1	1.6 ± 0.1[b]	54.2 ± 5.2[b]	14.6 ± 1.6	283 ± 28[b]	6.3 ± 0.9[b]	131 ± 46[b]
7	2.1 ± 0.1[c]	68.2 ± 6.1[c]	14.5 ± 2.3	219 ± 24[c]	12.4 ± 0.5[c]	168 ± 49[b]
12	1.9 ± 0.1[c]	71.2 ± 4.2[c]	14.6 ± 1.2	208 ± 21[c]	11.4 ± 1.7[c]	474 ± 77[c]
18	1.9 ± 0.1[c]	66.7 ± 6.8[c]	12.3 ± 2.2	177 ± 22[d]	12.5 ± 1.9[c]	573 ± 144[c]
Female						
1	1.6 ± 0.1[b]	55.3 ± 2.9[b]	15.1 ± 1.9	267 ± 22[b]	8.7 ± 2.4[b]	163 ± 41[b]
7	1.8 ± 0.1[c]	64.4 ± 4.1[c]	13.2 ± 1.0	206 ± 12[c]	14.3 ± 0.4[c]	319 ± 56[c]
18	1.8 ± 0.1[c]	71.8 ± 8.5[c]	14.7 ± 2.2	196 ± 8[c]	15.3 ± 2.1[c]	575 ± 73[d]

[a]Values are means ± SD of six rats per each age group. Means not followed by the same letter, [b,c] or [d], are significantly different ($P < 0.001$) within each sex.

TABLE 2

Changes in Lipid Composition and in α-Tocopherol and PCOOH Contents of Rat Liver upon Aging[a]

Rats (mon)	Liver weight (g)	Total lipids (TL) (mg/g brain)	PC (mg/g liver)	PC (mg/g TL)	α-Tocopherol (μg/g liver)	PCOOH (pmol/g liver)
Male						
1	7.2 ± 0.8[c]	34.4 ± 2.1	15.1 ± 1.7[c]	468 ± 10[c]	11.2 ± 3.1	274 ± 74[c]
7	21.9 ± 3.9[d]	30.0 ± 3.8[c]	13.3 ± 2.5	433 ± 52[c]	15.2 ± 2.5[c]	674 ± 19[d]
12	24.1 ± 2.8[d,e]	38.0 ± 4.7[d]	12.3 ± 1.4	338 ± 19[d]	9.3 ± 2.3[d]	1983 ± 388[e]
18	28.7 ± 3.1[e]	33.0 ± 7.8	9.9 ± 1.1[d]	309 ± 64[d]	13.2 ± 0.9[c]	2558 ± 348[e]
Female						
1	5.6 ± 0.5[b,c]	31.2 ± 4.0[c]	13.4 ± 2.8	425 ± 32[c]	18.8 ± 3.9[b,c]	409 ± 26[b,c]
7	14.0 ± 1.9[b,d]	28.7 ± 5.2[c]	13.6 ± 1.2	449 ± 49[c]	33.3 ± 4.3[b,d]	1266 ± 172[b,d]
18	14.1 ± 2.2[b,d]	49.6 ± 7.0[b,d]	13.6 ± 1.6[b]	274 ± 41[d]	25.1 ± 3.6[b,c]	1917 ± 432[b,e]

[a]Values are means ± SD of six rats per each age group. Means not followed by the same letter, [c,d] or [e], are significantly different ($P < 0.001$) within each sex. Abbreviations as in Table 1.
[b]Significantly different from corresponding male rat at $P < 0.001$.

TABLE 3

Changes in Lipid Composition and in α-Tocopherol and PCOOH Contents of the Heart and Lung of Male Rats with Aging[a]

Rats (mon)	Tissue weight (g)	Total lipids (TL) (mg/g tissue)	PC (mg/g tissue)	PC (mg/g TL)	α-Tocopherol (μg/g tissue)	PCOOH (pmol/g tissue)
Heart						
1	0.6 ± 0.1[b]	23.0 ± 1.9[b]	9.6 ± 1.8	435 ± 70[b]	13.7 ± 1.9[b]	506 ± 152[b,c]
7	1.5 ± 0.1[c]	22.1 ± 1.8[b]	10.8 ± 0.6	491 ± 51	20.4 ± 0.5[c]	434 ± 39[b]
12	1.5 ± 0.1[c]	18.8 ± 0.9[c]	10.0 ± 0.8	527 ± 68	21.6 ± 1.9[c]	635 ± 129[c,d]
18	1.6 ± 0.1[c]	23.5 ± 6.8	10.0 ± 1.4	539 ± 28[c]	21.4 ± 2.1[c]	799 ± 175[d]
Lung						
1	0.9 ± 0.1[b]	25.6 ± 3.6	6.9 ± 0.9	292 ± 23[b]	18.3 ± 3.1	145 ± 43
7	1.8 ± 0.2[c]	24.6 ± 5.0	5.8 ± 0.8	230 ± 44[c]	22.0 ± 0.8[b]	125 ± 39
12	2.0 ± 0.2[c]	24.0 ± 4.9	5.6 ± 0.4	236 ± 46	18.8 ± 2.5	88 ± 40
18	2.3 ± 0.1[d]	20.5 ± 2.6	5.9 ± 0.5	268 ± 14	17.9 ± 1.3[c]	92 ± 37

[a]Values are means ± SD of six rats per each age group. Means not followed by the same letter, [b,c] or [d], are significantly different ($P < 0.001$) within each organ. See Table 1 for abbreviations.

contents of the heart and lung were not age-dependent. PC contents in the heart and lung were constant during the entire feeding period, except that a higher PC content in heart TL was observed for the 18-month-old rats. α-Tocopherol content in the heart and lung did not change with age. PCOOH levels in the heart increased slightly in the 18-month-old rats, but PCOOH levels in the lung did not increase.

Molar ratio of PC and PCOOH. Table 4 shows the changes in molar ratio of PC and PCOOH in rat tissues with aging. For both the brain and liver, age-dependent proportional increases in PCOOH/PC ratios were clearly evident in male and in female rats. The brain PCOOH/PC ratio for the 1-month-old male rats was 0.6×10^{-5} and that for the 18-month-old male rats was 3.5×10^{-5}. The rate of increase in brain PCOOH/PC ratio was essentially the same males and females. The liver PCOOH/PC ratio of 1-month-old male rats was 1.9×10^{-5} and that of 18-month-old male rats was 19.7×10^{-5}. A similar increase was observed for female liver, although the increase in PCOOH/PC ratio with time for the females was lower than that of the males. The change in PCOOH/PC ratio in the heart did not correlate with aging, and the lung PCOOH/PC ratio showed no change with aging.

Fatty acids composition. Table 5 shows the changes in fatty acid composition of brain TL and PC following aging in male rats. In the TL, oleic acid (18:1n-9), eicosenoic acid (20:1n-9) and docosatetraenoic acid (22:4n-6) levels in the 7–18-month-old rats were significantly higher than those in the 1-month-old rats. Docosahexaenoic

TABLE 4

Age-Dependent Changes in the Molar Ratio of Phosphatidylcholine (PC) to Phosphatidylcholine Hydroperoxide (PCOOH) in Rat Tissues[a]

Rats (mon)	PCOOH/PC (10^5 × ratio)[b] Brain	Liver	Heart	Lung
Male				
1	0.6 ± 0.1[d]	1.9 ± 0.2[d]	4.0 ± 0.5[d]	1.6 ± 0.2
7	1.0 ± 0.1[e]	4.5 ± 0.7[e]	3.0 ± 0.1[e]	1.7 ± 0.2
12	2.5 ± 0.2[f]	11.8 ± 0.7[f]	4.8 ± 0.4[d]	1.3 ± 0.2
18	3.5 ± 0.4[g]	19.7 ± 2.8[g]	6.1 ± 0.5[f]	1.2 ± 0.2
Female				
1	0.8 ± 0.1[d]	3.0 ± 0.2[d]	n.d.[c]	n.d.
7	1.8 ± 0.1[e]	7.1 ± 0.8[e]	n.d.	n.d.
18	3.0 ± 0.2[f]	11.3 ± 0.5[f]	n.d.	n.d.

[a]Values are means ± SD of six rats per each age group. Means not followed by the same letter, [d,e,f] or [g], are significantly different ($P < 0.001$) within each sex.
[b]Molor ratio was calculated for 1-palmitoyl-2-arachidonoyl PC and its monohydroperoxide.
[c]Not determined.

TABLE 5

Changes in Fatty Acid Composition of Brain Total Lipids (TL) and Phosphatidylcholines (PC) of Male Rats with Aging[a]

Rats (mon)	Fatty acid (wt%)[b]						
	16:0	18:0	18:1n-9	20:1n-9	20:4n-6	22:4n-6	22:6n-3
				TL			
1	23.9 ± 0.8	21.8 ± 1.1[c]	19.0 ± 0.8[c]	0.6 ± 0.1[c]	10.0 ± 1.3	2.2 ± 0.2[c]	11.3 ± 0.5[c]
7	22.6 ± 1.3	18.9 ± 0.9[d]	23.9 ± 0.8[d]	1.6 ± 0.2[d]	9.5 ± 0.5	2.8 ± 0.2[d]	10.0 ± 0.2[d]
12	23.5 ± 1.4	19.3 ± 1.1	24.4 ± 1.1[d]	2.0 ± 0.2[d]	9.2 ± 0.9	3.1 ± 0.2[d,e]	7.1 ± 0.8[e]
18	21.7 ± 1.3	19.6 ± 1.2	25.1 ± 1.2[d]	2.4 ± 0.5[d]	9.6 ± 0.7	3.2 ± 0.1[e]	7.0 ± 0.7[e]
				PC			
1	56.7 ± 2.1[c]	18.6 ± 1.3[c]	11.2 ± 0.9[c]	0.6 ± 0.1[c]	2.1 ± 0.3[c]	trace	0.5 ± 0.1[c]
7	51.1 ± 1.2[d]	16.7 ± 0.9[c]	15.3 ± 1.0[d]	1.4 ± 0.2[d]	1.6 ± 0.2[c]	trace	0.5 ± 0.1[c]
12	46.0 ± 2.4[e]	13.3 ± 0.8[d]	25.1 ± 1.1[e]	1.4 ± 0.2[d]	3.0 ± 0.2[d]	trace	1.1 ± 0.3[d]
18	42.1 ± 1.8[e]	13.7 ± 0.7[d]	26.8 ± 1.2[e]	1.6 ± 0.1[d]	3.1 ± 0.2[d]	trace	1.0 ± 0.3[d]

[a]Values are means ± SD of six rats per each age group. Means not followed by the same letter, [c,d] or [e], are significantly different ($P < 0.001$).
[b]Individual fatty acids are designated by the number of carbon atoms and the number of double bonds, n represents the position of the first double bond from the methyl end.

acid (DHA, 22:6n-3) content was lower in 12- and 18-month-old rats than in 1- and 7-month-old rats. Saturated fatty acid (16:0, 18:0) content remained constant during the 18-mon experimental period. In brain, PC decreased in saturated fatty acids (16:0, 18:0) and increased in unsaturated fatty acids (18:1, 20:1, 20:4, 22:6) with aging.

DISCUSSION

In the present study, we have demonstrated the accumulation of PCOOH in the brain and in the liver of rats with aging by use of a CL-HPLC method for hydroperoxide determination. Such an age-dependent increase in PCOOH was not observed in lung and heart. We also showed that PCOOH was present in all the internal organs we examined at levels of 100 to 2500 pmol per gram of tissue (Tables 1–4). These values are much lower than previous data reported for lipid peroxide levels (hundreds of nanomoles per gram tissue) based on TBA measurements (5–11).

In previous studies, age-related accumulation of lipid peroxides as measured by TBA assay was reported to be 2 or 3 times higher in rat brain (5,8,9,11,15) and liver (8,9,11,15), and 1.2 times higher in rat liver lipids based on conjugated diene measurements (15). In the present study, it was shown that the age-related formation of PCOOH in brain (3.5–4.4 times) and liver (4.7–9.3 times) was more pronounced than previously observed (5,8,9,15). From the evidence provided here based on data obtained on aging rodents, membrane phospholipids in brain and liver appear more susceptible to hydroperoxidation than the lipids in heart and lung.

We recently reported the presence of phosphatidylethanolamine hydroperoxide (PEOOH) in addition to PCOOH in rat brain and liver (20). In rat brain, PEOOH content was generally higher than PCOOH levels. Phosphatidylethanolamine in brain and liver is also richer in unsaturated fatty acids than is PC; hence, the present findings are not surprising. Liver phospholipid hydroperoxide content is affected by dietary supplementation, *e.g.*, with fish oil, which increases liver PCOOH and PEOOH levels (20).

An age-dependent decrease in phospholipid levels has been reported for rat liver (34), human brain (35), rat brain and liver microsomes (36,37) and for erythrocyte membranes (38). In the present study, a slight decrease in brain PC content (Table 1), as well as a decrease in brain PC content relative to brain TL, was evident (Table 1). The liver PC content was decreased whether expressed relative to liver weight or to liver TL (Table 2). This PC decrease relative to TL and the changes in phospholipid composition may have a significant effect on membrane fluidity (38,39) in these organs and thus might change membrane function as well (40). As shown in this study, the organs showing a decrease in PC content were the brain and the liver, and only in these two organs was an age-dependent accumulation of PCOOH observed. The PC decrease may have resulted from peroxidative breakdown of PC as well as from changes in phospholipid metabolism that may occur during aging. The changes in phospholipid composition also seem to directly correlate with the susceptibility to hydroperoxidation of the membrane phospholipids themselves (40,41).

The content of α-tocopherol as one of the antioxidants in biomembranes did not decrease in the brain and liver upon aging (Tables 1 and 2), although PCOOH had significantly accumulated in these two organs. It thus appears that α-tocopherol cannot effectively function as an antioxidant and inhibit PC hydroperoxidation due to aging in brain or liver. It could alternatively suggest that higher α-tocopherol levels may be required to prevent phospholipid hydroperoxidation in the membranes of brain and liver during aging, as was suggested by Meydani *et al.* (42). Upon aging, an increase in superoxide radical formation (8) and a decrease in superoxide dismutase activity, catalase activity (11) and glutathione content (9) have been reported to occur in rat brain and in rat liver. The increase in PCOOH in rat brain and liver with age, as shown here, should also affect membrane association and activation of protein kinase C. It has been reported that oxidative modification of membrane lipids can result in persistent activation of protein kinase C (43,44).

The livers of 18-month-old male rats showed 10.4-fold higher PCOOH/PC ratios than 1-month-old rats (Table 4).

This enhanced PCOOH/PC molar ratio in liver lipids of the aged rats was however, significantly lower than that reported for the liver of mice (32.3×10^{-5}) fed a hepatocarcinogenic choline-deficient diet containing ethionine (24).

DHA content decreased in TL of the brain with aging. On the other hand, arachidonic acid and DHA content significantly increased in PC in aged brain. Brain PC containing arachidonic acid or DHA would be expected to be sensitive to hydroperoxidation in the course of aging.

In the present study, we have demonstrated that PCOOH accumulates as a primary peroxidation product of membrane phospholipids in brain and liver of the rat upon aging. These findings provide important evidence that oxidative damage occurs to membrane phospholipids in the course of senescence and aging as shown in this *in vivo* system.

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Modulation of Phosphatidylcholine Biosynthesis by Peroxisome Proliferating Fatty Acid Analogues

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The modulation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) biosynthesis by sulfur-substituted fatty acid analogues has been investigated in rats. We have compared the effects of two non-β-oxidizable fatty acid analogues, 3-thiadicarboxylic acid and tetradecylthioacetic acid, which induce proliferation of peroxisomes, with those of the analogue tetradecylthiopropionic acid, which is a weak peroxisome proliferator. Repeated administration of 3-thiadicarboxylic acid for seven days resulted in increased hepatic concentrations of both PC and PE, but the PC/PE ratio was decreased. PC synthesis was increased, as evidenced by increased incorporation of [^{3}H]choline into PC and an increased activity of cytidinetriphosphate (CTP): phosphocholine cytidylyltransferase. This was accompanied by a reduction in the pool sizes of choline and phosphocholine. The *S*-adenosylmethione/*S*-adenosylhomocysteine ratio (AdoMet/AdoHcy) was marginally affected, indicating no increase in the rate of methylation of PE to PC. Administration of tetradecylthioacetic acid also resulted in increased hepatic phospholipid levels, increased AdoMet/AdoHcy ratios and in slightly elevated activity of CTP:phosphocholine cytidylyltransferase. The most striking effect observed after tetradecylthiopropionic acid treatment was the development of fatty liver. The activity of CTP:phosphocholine cytidylyltransferase and the incorporation of [^{3}H]choline into PC was reduced compared to 3-thiadicarboxylic acid treatment. Although the rate of methylation of PE seemed to be increased at an elevated AdoMet/AdoHcy ratio, this resulted in only minor changes in the hepatic PC and PE levels, and the PC/PE ratio remained unchanged. Furthermore, the hepatic levels of choline and phosphocholine were reduced in these rats. The activities of the two enzymes competing for choline in the liver, choline kinase and choline dehydrogenase were changed in opposite directions, with the activity of choline kinase increasing approximately 1.5-fold. In addition, it was found that the level of homocysteine was elevated in the liver of tetradecylthiopropionic acid-treated rats. The possibility is discussed that this reflects a reduced flux of choline through the oxidative pathway in the liver. In tetradecylthiopropionic acid-treated rats, there seemed to be a coordinated regulation of the two pathways for PC biosynthesis, with an increase in the methylation of PE to PC and a reduced synthesis *via* the CDPcholine pathway. The increase in PC observed in rats treated with 3-thiadicarboxylic acid and tetradecylthioacetic acid suggests that increased PC synthesis is linked to peroxisome proliferation.

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3-Thiadicarboxylic acid [HOOC-CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH] and tetradecylthioacetic acid [CH_3-$(CH_2)_{13}$-S-CH_2-COOH] have been shown to be lipid-lowering agents and to decrease triacylglycerol, cholesterol and phospholipid levels in plasma (1–3). In addition, these agents are strong peroxisome proliferators, the dicarboxylic acid being the most potent (1). Both peroxisomal and mitochondrial β-oxidation are stimulated by these analogues (3–5). Tetradecylthiopropionic acid [CH_3-$(CH_2)_{13}$-S-CH_2-CH_2-COOH] is a weak peroxisome proliferator which inhibits mitochondrial β-oxidation, leading to the development of fatty liver. The agent is hypolipidemic when fed to rats in relatively high doses (2–4).

Phosphatidylcholine (PC) is the major phospholipid present in plasma lipoproteins and in liver (6). PC is synthesized mainly through the cytidinediphosphate(CDP)choline pathway (*de novo* pathway), but in the liver PC may also be synthesized by methylation of phosphatidylethanolamine (PE) (7). It is widely accepted that in the former pathway the rate-limiting step is the formation of CDPcholine from cytidinetriphosphate (CTP) and phosphocholine catalyzed by CTP:phosphocholine cytidylyltransferase (CPCT). It has been postulated that the activity of this enzyme is regulated by translocation of the inactive cytosolic form to the active microsomal form (8). The biosynthesis of PC may, however, be increased by an increase in the total activity of CPCT without any changes in the relative distribution of the enzyme (9,10). We have previously reported that sulfur-substituted fatty acid analogues modulate the activities of microsomal and cytosolic CPCT to the same extent (5). The enzyme was not translocated from the cytosolic to the microsomal fraction *in vivo*, although translocation of CPCT has been observed *in vitro* (11).

The methylation of PE to PC is catalyzed by PE *N*-methyltransferase, an enzyme associated with the microsomal fraction from rat liver. This enzyme is mainly regulated by the availability of the substrates PE and *S*-adenosylmethionine (AdoMet) (7) and, more importantly, the methylation of PE is modulated by the ratio between AdoMet and *S*-adenosylhomocysteine (AdoHcy) (12). Several studies have pointed to a coordinated control of the methylation pathway and the CDPcholine pathway, *i.e.*, one pathway is upregulated if the other is downregulated (7), although this is not always the case (13).

We wanted to extend our earlier studies on the effect of sulfur-substituted fatty acid analogues on the metabolism of phospholipids in rat liver and, in particular, further explore the differences between analogues which are strong and weak peroxisome proliferators. During the process of peroxisome proliferation, there must be an increase in membrane biosynthesis and, therefore, an increased demand for phospholipids. The more potent peroxisome proliferators increase the oxidation of fatty acids in contrast to the fatty

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Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; CPCT, cytidinetriphosphate (CTP):phosphocholine cytidylyltransferase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)N,N′-tetraacetic acid; FID, flame-ionization detector; Hcy, homocysteine; HEPES, N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

liver-promoting analogue, which inhibits the oxidation of fatty acids. The supply of fatty acids for glycerolipid synthesis may thus be quite different with these two groups of agents, which might in turn influence the biosynthesis of PC. In addition, it was of interest to examine the relative contribution of the two pathways in the synthesis of PC and to determine to what extent these pathways are coordinatedly regulated.

MATERIALS AND METHODS

Chemicals and drugs. 3-Thiadicarboxylic acid, tetradecylthioacetic acid and tetradecylthiopropionic acid were prepared as described earlier (3,14). *S*-Adenosyl-L-[*methyl*-^{3}H]methionine (62 Ci/mmol), [*methyl*-^{3}H]choline chloride (76 Ci/mmol), [*methyl*-^{14}C]choline chloride (55 mCi/mmol), CDP[*methyl*-^{14}C]choline (50 mCi/mmol) and phosphoryl-[*methyl*-^{14}C]choline (50 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England), and CDP[*ethanolamine*-1,2-^{14}C] (52 mCi/mmol) from ICN (Costa Mesa, CA). Choline chloride, CDPcholine, CDPethanolamine, phosphorylcholine, 1,2-dioleoyl-*sn*-glycerol and choline kinase were obtained from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase was from Boehringer (Mannheim, Germany). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and treatments. Male Wistar rats from Møllegaard Breeding Laboratory (Ejby, Denmark) weighing 150–200 g were housed individually in metal wire cages in a room maintained at a 12-h light-dark cycle and at a constant temperature of 20 ± 3°C. The animals were acclimatized for at least 5 d under these conditions before the start of the experiments. The fatty acid analogues were suspended in 0.5% sodium carboxymethyl cellulose and administered by gastric intubation in a volume of 0.7–1.0 mL once a day. The control animals received only sodium carboxymethyl cellulose. All animals had free access to water and food. At the end of the experiments, the fasted rats (12 h) were lightly anesthetized and cardiac puncture was performed. The livers were removed and immediately chilled on ice and weighed.

In experiments with [*methyl*-^{3}H]choline chloride, rats were lightly anesthetized and the radioactive isotope (40 μCi in 0.4 mL saline) was injected through the tail vein. Rats were killed 15 min later and 1 g of fresh liver was immediately extracted with 20 mL of chloroform/methanol (2:1, vol/vol) (15). The incorporation of labeled choline into PC was determined by subjecting an aliquot of the washed chloroform phase to silicic acid thin-layer chromatography (TLC) in chloroform/metanol/acetic acid/water (50:30:7:3, by vol.) The bands were visualized with I_2 vapor, and the bands corresponding to PC were scraped into liquid scintillation vials and counted.

Preparation of total homogenate and cellular fractions. The livers from individual rats were homogenized in ice-cold sucrose medium [0.25 M sucrose in 10 mM *N*-(2-hydroxyethyl)piperacine-*N'*-(2-ethanesulfonic acid) (HEPES) buffer, pH 7.4] and 2 mM ethylenediaminetetraacetic acid (EDTA). The postnuclear fraction was used for further analysis by differential centrifugation. Samples from three animals were pooled and mitochondria-enriched, peroxisome-enriched, and microsomal and cytosolic fractions were isolated (1).

Other analytical methods. The activity of cholinephosphotransferase was measured in the microsomal fraction as described by Weinhold *et al.* (16), with some modifications. The assay mixture contained 50 mM Tris/HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM ethyleneglycol-*bis*(β-aminoethyl ether(*N,N'*-tetraacetic acid (EGTA), 1 mM 1,2-dioleoyl-*sn*-glycerol and 0.2 mg/mL Tween 20. The mixture was sonicated, microsomes (0.2 mg/mL) and CDP[*methyl*-^{14}C]choline (1 mM) were added, and the reaction mixture was incubated for 15 min at 37°C. Ethanolaminephosphotransferase activity was assayed by an identical assay except for the use of CDP-[*ethanolamine*-1,2-^{14}C]ethanolamine as substrate instead of CDP[*methyl*-^{14}C]choline. Choline dehydrogenase was assayed oxygraphically in the mitochondrial fraction as described previously (17). Choline kinase was assayed in the cytosolic fraction with [*methyl*-^{14}C]choline chloride as substrate (18). CPCT was assayed in the cytosolic and in the microsomal fraction with phosphoryl[*methyl*-^{14}C]choline as substrate (19). PE *N*-methyltransferase activity was measured in the microsomal fraction as incorporation of [*methyl*-^{3}H] from *S*-adenosyl-L-[*methyl*-^{3}H]methionine into PE (20), and the products were isolated and quantitated by TLC as described by Schanche *et al.* (21).

The amounts of choline and phosphocholine were determined in the water-soluble fraction after lipid extraction of the liver. Aliquots were analyzed as described by Post *et al.* (22), except that the column (Dowex AG1-X8) was eluted as described in the assay for choline kinase. The level of homocysteine (Hcy) in the liver was determined with a radio-enzymic assay (23), whereas AdoHcy and AdoMet were determined in an acid extract by high-performance liquid chromatography (HPLC) (24).

Total phospholipid content was determined using the Monotest phospholipids enzymatic kit (Boehringer). Hepatic levels of PE and PC were quantitated using an Iatroscan TH-10 TLC + FID (latron) instrument. One-μL samples were spotted on Chromarods consisting of sintered layers precoated with silica gel. The rods were developed first in ethyl acetate/isooctane/glacial acetic acid/water (9:5:2:10, by vol; upper phase) and then in chloroform/methanol/40% methylamine (120:35:5, by vol), and the resulting chromatograms were analyzed using software from Boreal (Flotec, France). Standard curves for PE and PC were used in calculating the amounts of phospholipids present in the samples.

Statistical analysis. The results are presented as means ± SD from three animals unless otherwise stated. Enzymatic activities were determined in pooled fractions from three treated animals, and in this case the results are given only as means. Control activities were determined in nine to twelve animals and are given as means ± SD. Differences were tested by a two-way analysis of variance at 95% confidence interval using a statistical software package (StatView SE; Abacus Concepts Inc., Brain Power Inc., Calabasas, CA).

RESULTS

Plasma and liver phospholipid levels. Administration of the hypolipidemic fatty acid analogues, 3-thiadicarboxylic acid and tetradecylthioacetic acid significantly lowered plasma phospholipid levels consistent with what has been observed previously (1) (Table 1). These two analogues at

TABLE 1

Hepatic and Plasma Phospholipid Levels in Rats Fed Sulfur-Substituted Fatty Acid Analogues for Seven Days[a]

Treatment	Dose (mg/d/kg body weight)	Hepatic phospholipids (μmol/g liver)	Plasma phospholipids (μmol/mL)
Control	0	19.0 ± 1.4	1.50 ± 0.18
3-Thiadicarboxylic acid	75	21.1 ± 0.2[b]	1.10 ± 0.15[b]
	250	22.6 ± 0.4[b]	0.82 ± 0.30[b]
Tetradecylthioacetic acid	75	21.6 ± 1.8	1.05 ± 0.25
	250	23.2 ± 2.2[b]	0.90 ± 0.15[b]
Tetradecylthiopropionic acid	75	19.5 ± 1.1	1.60 ± 0.15
	250	20.1 ± 0.9	1.90 ± 0.10[b]

[a]The tabulated values are the means ± SD of twelve control animals and three rats in each experimental group.
[b]$P < 0.05$ for difference between control and treated rats.

the highest dose used also increased hepatic phospholipid levels by approximately 15–20%. The agents have also been shown to cause moderate hepatomegaly increasing relative liver weights by 30–40% after 7 d of feeding (1,4). Taking this into account, the increase in liver phospholipids is actually greater than seen from Table 1. Tetradecylthiopropionic acid, on the other hand, only marginally affected hepatic phospholipid levels, but increased plasma phospholipid levels.

To further elucidate the effect of the fatty acid analogues on phospholipid metabolism, an experiment was carried out in which rats were fed 3-thiadicarboxylic acid and tetradecylthiopropionic acid for seven days. The rats were then injected intravenously with [^{3}H]choline and killed 15 min later. Hepatic PC levels and PE levels were measured, and as can be seen from Table 2 the levels of both changed in the same direction. 3-Thiadicarboxylic acid increased both the PC and PE content, and in these rats the rate of synthesis of PC increased as shown by a higher incorporation of [^{3}H]choline into PC as compared to controls and to tetradecylthiopropionic acid-treated rats. In contrast, no significant changes were observed in the hepatic concentrations of PC and PE and in the incorporation of [^{3}H]choline into PC after tetradecylthiopropionic acid administration (Table 2). It is worth noting, however, that the changes observed in the PC and PE levels were not of the same order, resulting in a decreased ratio of PC to PE in the 3-thiadicarboxylic acid-fed animals. Tetradecylthiopropionic acid treatment, on the other hand, tended to increase the ratio between PC and PE compared to 3-thiadicarboxylic acid-treated rats (Table 2).

Regulation of the CDPcholine pathway. The enzyme choline kinase catalyzes the phosphorylation of choline, the first step in the CDPcholine pathway of PC biosynthesis. This enzyme competes for choline with choline dehydrogenase, which catalyzes the oxidation of choline to betaine (25). Betaine acts as a methyl donor in the conversion of Hcy to methionine in the liver (Fig. 1).

The activities of these two enzymes have been measured in liver of rats treated with the different fatty acid analogues. Tetradecylthiopropionic acid increased the activity of choline kinase (Table 3), whereas all three analogues decreased the activity of choline dehydrogenase. More informative, however, is to compare the ratio of choline dehydrogenase and choline kinase activities (Fig. 2). This ratio was decreased after administration of tetradecylthiopropionic acid. After seven days of feeding this analogue, the ratio was decreased nearly threefold, indicating a change in the choline flux away from the oxidative pathway. In contrast, 3-thiadicarboxylic acid and tetradecylthioacetic acid marginally elevated the ratio between choline dehydrogenase and choline kinase activities.

TABLE 2

Changes in Hepatic Levels of Phospholipids, Phosphocholine and Choline and in the Incorporation of [^{3}H]Choline into Phosphatidylcholine in Liver of Rats Treated with Fatty Acid Analogues for Seven Days at a Dose of 150 mg/d/kg Body Weight[a]

	Control	3-Thiadicarboxylic acid	Tetradecylthiopropionic acid
PC (mg/g liver)	11.33 ± 0.81	13.42 ± 1.01[b]	10.51 ± 1.07[c]
PE (mg/g liver)	5.90 ± 0.48	7.72 ± 1.10[b]	5.23 ± 0.72[c]
Ratio (PC/PE)	1.94	1.76	2.02
Choline (μmol/g liver)	0.114 ± 0.025	0.053 ± 0.016[b]	0.072 ± 0.025[b]
Phosphocholine (μmol/g liver)	1.54 ± 0.21	1.12 ± 0.16[b]	1.19 ± 0.28[b]
Incorporation of [^{3}H]choline into PC (cpm/15 min/g liver)	4783 ± 670	8415 ± 1570[b]	4560 ± 990[c]

[a]The tabulated values are the means ± SD of four to five rats in each experimental group. Phosphatidylcholine, PC; phosphatidylethanolamine, PE.
[b]$P < 0.05$ for difference between control and treated rats.
[c]$P < 0.05$ for difference between the two treatment groups.

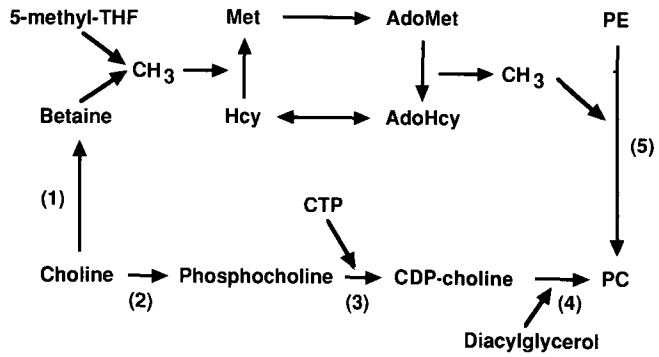

FIG. 1. Interrelationship between choline, methionine and phosphatidylcholine metabolism; THF, tetrahydrofolate; Met, methionine; AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine; Hcy, homocysteine; CTP, cytidinetriphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; (1), choline dehydrogenase; (2), choline kinase; (3), CTP:phosphocholine cytidylyltransferase; (4), cholinephosphotransferase; (5), PE *N*-methyltransferase.

We have previously reported that CPCT activity is elevated in rats treated with 3-thiadicarboxylic acid and tetradecylthioacetic acid, in which hepatic phospholipid levels are also increased. The activity changed in parallel fashion in the microsomal and the cytosolic fractions (5). This also can be seen from Tables 2 and 3, where the hepatic PC levels and the changes in the total enzyme activities are tabulated. In contrast, the total CPCT activity was reduced by approximately 25% in tetradecylthiopropionic acid-trated rats (Table 2).

The activity of the last enzyme in the CDPcholine pathway, cholinephosphotransferase, was only marginally affected by tetradecylthiopropionic acid for up to 7 d of feeding, but was stimulated by 25% after 14 d. 3-Thiadicarboxylic acid increased cholinephosphotransferase activity after administration for a few days, whereas tetradecylthioacetic acid decreased it by more than 25% after only one day of feeding (Fig. 3).

Hepatic choline and phosphocholine levels were also changed in rats fed the fatty acid analogues. 3-Thiadicarboxylic acid reduced the levels of both significantly, and tetradecylthiopropionic acid feeding tended to decrease the pool sizes of both metabolites, although to a lesser extent than did the dicarboxylic acid (Table 2).

PE synthesis and the methylation pathway to PC. PE is synthesized *via* the CDPethanolamine pathway, which parallels the CDPcholine pathway. The final enzyme in this pathway, ethanolaminephosphotransferase, catalyzes the transfer of CDPethanolamine to 1,2-diacyl-*sn*-glycerol. The enzyme PE *N*-methyltransferase can then *N*-methylate phosphatidylethanolamine by successive transfer of methyl groups from AdoMet, giving the final product PC (Fig. 1). Repeated administration of 3-thiadicarboxylic

TABLE 3

Changes in Relative Specific Activities of Enzymes Involved in Choline Metabolism in Rats Treated with Fatty Acid Analogues for Seven Days at a Dose of 150 mg/d/kg Body Weight[a]

Enzyme	3-Thiadicarboxylic acid	Tetradecylthioacetic acid	Tetradecylthiopropionic acid
Choline dehydrogenase	83.1	86.2	69.1
Choline kinase	78.3	67.7	152.0
CPCT (total)	122.0	109.3	75.2
Cholinephosphotransferase	116.7	75.6	105.0

[a]The enzyme activities represent the means of pooled fractions from three treated animals and are presented relative to those of nine control animals (see Materials and Methods). Control values (100%): Choline kinase, 42.7 ± 5.8; cytidinetriphosphate:phosphocholine cytidylyltransferase (CPCT), 53.8 ± 5.3; cholinephosphotransferase, 249.45 ± 21.6; choline dehydrogenase, 396.2 ± 35.0 nmol/min/g liver.

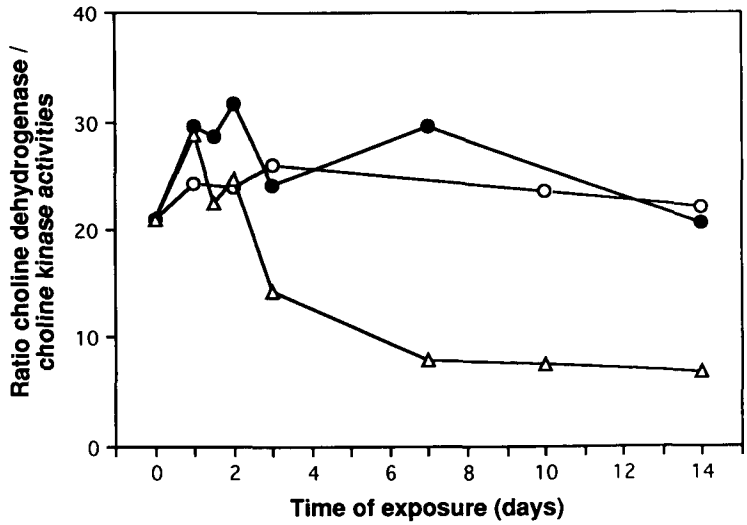

FIG. 2. Effect of 3-thiadicarboxylic acid (○), tetradecylthioacetic acid (●), and tetradecylthiopropionic acid (△) on the ratio of choline dehydrogenase and choline kinase activities. Rats were fed fatty acid analogues at a dose of 150 mg/day/kg body weight. Enzyme activities were determined in pooled fractions from three animals as described in Materials and Methods.

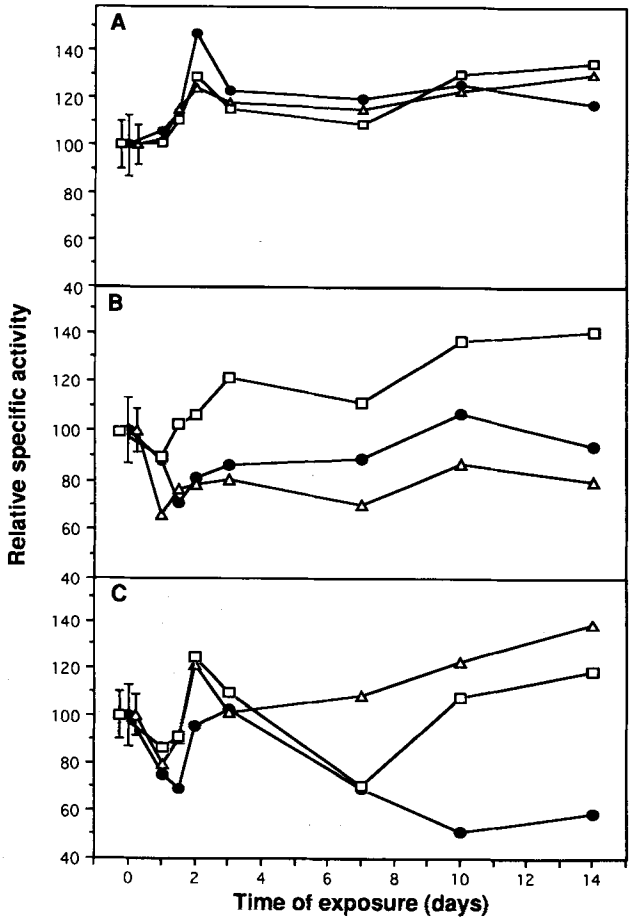

FIG. 3. Effect of 3-thiadicarboxylic acid (A), tetradecylthioacetic acid (B), and tetradecylthiopropionic acid (C) on the activities of phosphatidylethanolamine (PE) *N*-methyltransferase (●), cholinephosphotransferase (△), and ethanolaminephosphotransferase (□). Rats were fed fatty acid analogues at a dose of 150 mg/d/kg body weight. The enzyme activities represent the means of pooled fractions from three treated animals and are presented relative to those of nine control animals (see Materials and Methods). Control values (100%); PE *N*-methyltransferase, 4.23 ± 0.53; cholinephosphotransferase, 249.45 ± 21.6; ethanolaminephosphotransferase, 71.95 ± 7.2 nmol/min/g liver.

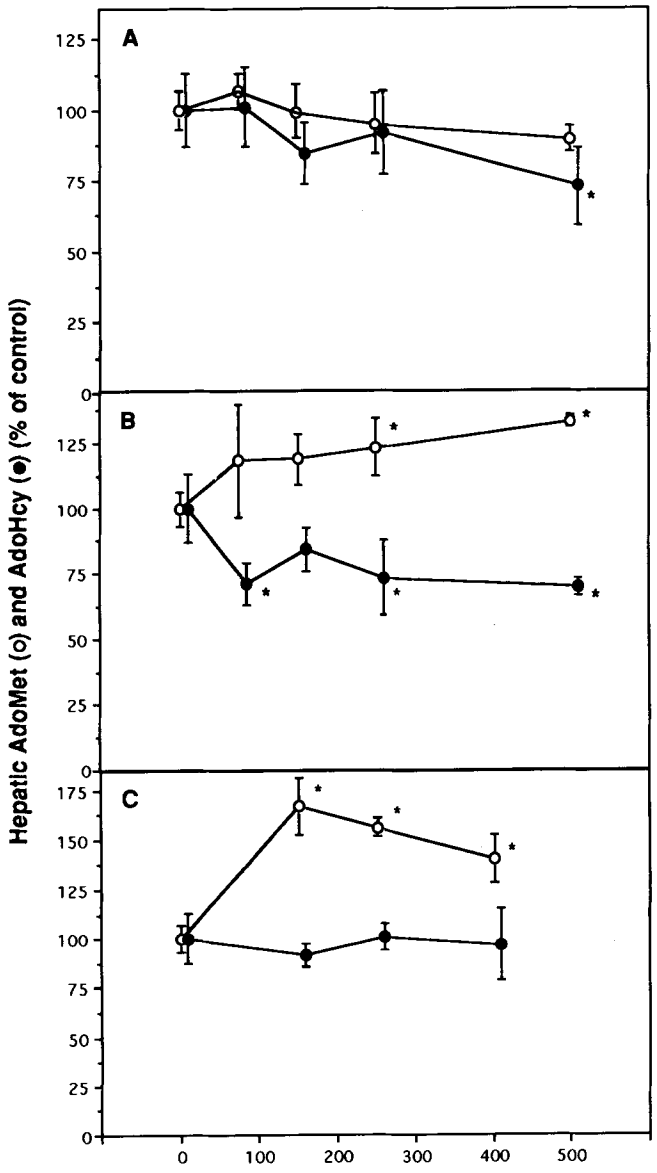

FIG. 4. Effect of 3-thiadicarboxylic acid (A), tetradecylthioacetic acid (B), and tetradecylthiopropionic acid (C) on hepatic AdoMet (○) and AdoHcy (●) levels. Rats were fed fatty acid analogues for seven days. The tabulated values are the means ± SD of twelve control animals and three rats in each experimental group. *$P < 0.05$ for difference between control and treated rats. Control values (100%); AdoMet, 151.1 ± 10.1; AdoHcy, 20.7 ± 2.6 nmol/g liver. Abbreviations as in Figure 1.

acid significantly enhanced the level of PE (Table 2). The activities of ethanolaminephosphotransferase and PE *N*-methyltransferase were both increased, an effect that was apparent after two days of feeding (Fig. 3). Tetradecylthioacetic acid had the same effect on the former enzyme, whereas the activity of PE *N*-methyltransferase was essentially unchanged. In contrast, the fatty liver producing analogue, tetradecylthiopropionic acid, decreased the activity of the latter enzyme; PE *N*-methyltransferase activity was decreased by more than 50% after prolonged feeding.

AdoMet functions as a methyl donor in cells, which provides methyl groups for a variety of acceptors, including PE. AdoHcy, the by-product in these reactions, is hydrolyzed to Hcy. Hcy can then be remethylated to methionine, obtaining methyl groups either from 5-methyltetrahydrofolate or from betaine (Fig. 1).

Repeated administration of 3-thiadicarboxylic acid slightly decreased the hepatic level of both AdoMet and AdoHcy (Fig. 4). Calculation of the AdoMet/AdoHcy ratio showed that this parameter was unchanged (Table 4). Tetradecylthioacetic acid increased the hepatic level of AdoMet, amounting to a 40% increase at the highest doses used, while the AdoHcy level was decreased. Overall, this resulted in an increased AdoMet/AdoHcy ratio (Table 4). Tetradecylthiopropionic acid also increased the AdoMet/AdoHcy ratio, in this case mainly due to an increase in AdoMet. The ratio increased by nearly 70% at a dose of 150 mg/d/kg body weight (Fig. 4).

The level of Hcy was increased in liver (Table 4) and plasma (data not shown) after feeding tetradecylthiopro-

TABLE 4

Effect of Sulfur-Substituted Fatty Acid Analogues on the Amount of Sulfur-Containing Metabolites in Liver and Plasma of Rats Fed Fatty Acid Analogues for Seven Days[a]

Treatment	Dose (mg/d/kg body weight)	Ratio AdoMet/AdoHCy	Homocysteine (nmol/g liver)
Control	0	7.4 ± 1.0	6.3 ± 1.1
3-Thiadicarboxylic acid	150	8.7 ± 1.3[b]	n.d.
	250	7.8 ± 1.8[c]	8.6 ± 1.3[b]
Tetradecylthioacetic acid	150	10.4 ± 0.7[d]	9.9 ± 2.5
	250	10.8 ± 1.7[d]	7.5 ± 2.2
Tetradecylthiopropionic acid	150	13.4 ± 1.1[d]	n.d.
	250	11.4 ± 0.7[d]	13.3 ± 2.4[d]

[a]The tabulated values are the means ± SD of nine control animals and three rats in each experimental group. n.d., not determined. AdoMet, *S*-adenosylonethionine; AdoHCy, *S*-adenosylhomocysteine.
[b]$P < 0.05$ for different means from tetradecylthiopropionic acid-treated rats.
[c]$P < 0.05$ for different means from tetradecylthioacetic acid- and tetradecylthiopropionic acid-treated rats.
[d]$P < 0.05$ for different means from the control rats.

pionic acid, while 3-thiadicarboxylic acid and tetradecylthioacetic acid tended to increase the hepatic homocysteine levels, although not significantly.

DISCUSSION

In this study we have investigated the modulation of phospholipid metabolism in the liver of rats fed different fatty acid analogues. The fatty acid analogues can conveniently be divided into two groups. 3-Thiadicarboxylic acid and tetradecylthioacetic acid act as strong peroxisome proliferators and have hypolipidemic effects. Tetradecylthiopropionic acid is a weak peroxisome proliferator (1,4). The most striking effect observed in rats treated with this analogue is the formation of fatty liver. The peroxisome proliferating analogues increase hepatic phospholipid levels (Table 1), which may be rationalized by an increased demand for membrane biosynthesis. Tetradecylthiopropionic acid, as a very weak peroxisome proliferator, does not elevate hepatic phospholipid levels.

The strongest peroxisome proliferator, 3-thiadicarboxylic acid, increases hepatic levels of both PC and PE (Table 2). The PC/PE ratio decreased, however, as the increase in PE was greater than the increase in PC. The same phenomenon has been observed in rats treated with other peroxisome proliferators, such as di(2-ethylhexyl)phthalate (26). The higher PC level may be due either to increased synthesis *via* the CDPcholine pathway or to increased methylation of PE. 3-Thiadicarboxylic acid increased the choline flux through the CDPcholine pathway, as evidenced by a higher incorporation of [^{3}H]choline into PC (Table 2). It can also be seen that the pool sizes of choline and phosphocholine were decreased, suggesting an increased utilization of these metabolites. The amount of [^{3}H]choline incorporated into PC is probably an overestimation of the rate of PC synthesis, as the decreased pool size of choline will increase the specific activity of [^{3}H]choline in the 3-thiadicarboxylic-treated rats. However, the incorporation of choline was clearly higher than in rats administered tetradecylthiopropionic acid as the choline pool was also decreased in these rats. The activity of the rate-limiting enzyme in the CDPcholine pathway, CPCT (microsomal plus cytosolic) was increased (Table 3), again suggesting an increased PC synthesis *via* this pathway. The activity was increased both in the microsomal and in the cytosolic fraction (5). Studies with partially hepatectomized rats (10) provided results very similar to those obtained in the 3-thiadicarboxylic-treated rats. In hepatectomized rats the increase in total CPCT activity was found to be due to increased enzyme mass as shown by immunotitration.

The increase in hepatic PC in 3-thiadicarboxylic acid treated rats may also result from increased methylation of PE. The PE *N*-methyltransferase activity measured *in vitro* was increased (Fig. 3), which may be related to the relative increase in PE in the membranes (Table 2). It has been reported that the level of PE in the membrane may influence the activity of this enzyme *in vitro*, while the *in vivo* activity was shown to be dependent on the availability of substrates (27). The amount of AdoMet was slightly decreased, and the ratio AdoMet/AdoHcy did not change significantly (Table 4). Altogether, this points to only small changes in the rate of methylation of PE in the 3-thiadicarboxylic-treated rats.

The weak peroxisome proliferator, tetradecylthiopropionic acid, had only minor effects on the levels of PC and PE, which were slightly, although not significantly, reduced. The incorporation of [^{3}H]choline into PC was well below what was found in 3-thiadicarboxylic acid-treated rats (Table 2), while the choline pool tended to be decreased. The decrease in the activity of CPCT (Table 3) points to a reduced synthesis of PC *via* the CDPcholine pathway. It can also be seen that the ratio of the activities of the choline metabolizing enzymes choline dehydrogenase and choline kinase was greatly reduced (Fig. 2), mainly due to a 1.5-fold increase in choline kinase activity. Choline kinase has an apparent K_m value for choline that is much smaller than the corresponding value for choline dehydrogenase (17,28). The observed changes may thus reflect a change in the choline flux away from the oxidative pathway and into the CDPcholine pathway in tetradecylthiopropionic acid-treated rats. However, this seems not to be sufficient to prevent a reduced synthesis of PC by this pathway. The hepatic level of AdoMet and the AdoMet/Ado-Hcy ratio are, however, elevated in these rats (Fig. 4). This could result in increased methylation of PE

to PC (7) to compensate for the decrease in PC synthesis *via* the CDPcholine pathway. In a recent report (29) in which the diurnal variation of PE methylation was described, a high AdoMet/AdoHcy ratio was found to correlate with a high PC/PE ratio, suggesting a high flux of methyl groups from AdoMet to PE under these conditions.

It has been reported that ethionine, a structural analogue of methionine, changes the choline flux in much the same way as does tetradecylthiopropionic acid (30). Both ethionine and tetradecylthiopropionic acid treatment result in a substantial increase in the hepatic level of Hcy and cause fat accumulation in the liver (31). The increase in the hepatic Hcy level observed in tetradecylthiopropionic acid-treated rats (Table 4) could thus be due to the reduced flux of choline through the oxidative pathway, thereby reducing the availability of methyl groups for methylation of homocysteine to methionine (Fig. 1). The higher AdoMet level observed in the same rats might contribute to this effect, as AdoMet may act as an allosteric inhibitor of 5,10-methylenetetrahydrofolate reductase (32), thereby reducing the level of 5-methyltetrahydrofolate, which is the other possible methyl donor for methylation of Hcy.

The AdoMet/AdoHcy ratio is also elevated in tetradecylthioacetic acid-treated rats (Table 4), and the total activity of CPCT is increased slightly (Table 3). The increase in hepatic PC content observed in these rats might thus be due both to an enhanced rate of methylation of PE and to a slight stimulation of the CDPcholine pathway.

The results presented in this study are in accordance with CPCT being the rate-limiting enzyme in the synthesis of PC *via* the CDP-choline pathway. Tetradecylthiopropionic acid is the only analogue which does not increase the hepatic phospholipid level, and is also the only analogue that does not increase the CPCT activity. The other two analogues increase the activity of both the microsomal and cytosolic cytidylyltransferase; translocation of the enzyme from the cytosolic to the microsomal fraction could not be observed (10).

In tetradecylthiopropionic acid-treated rats there seems to be a coordinated regulation of the two pathways for PC synthesis, the increase in the methylation of PE compensating for a reduced flux through the CDPcholine pathway. The possibility should be considered that limited availability of choline could increase the flux through the methylation pathway, thereby stimulating *de novo* synthesis of choline. In 3-thiadicarboxylic acid- and tetradecylthioacetic acid-treated rats, where hepatic phospholipid levels are elevated, the methylation of PE to PC seems to be increased if the CDPcholine pathway cannot supply the required amount of PC.

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Distribution of Ubiquinone and Ubiquinol Homologues in Rat Tissues and Subcellular Fractions

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The oxidized (UQ_{ox}) and reduced (UQ_{red}) forms of ubiquinone (UQ) homologues in rat tissues and subcellular fractions were analyzed to elucidate their distribution and physiological role. UQ-9 and UQ-10 were detected in all tissues studied, and UQ-9 was the predominant homologue. The total amount of UQ_{ox}-10 and UQ_{red}-10 was 20–50% that of UQ_{ox}-9 and UQ_{red}-9. The levels of these homologues were highest in heart with lesser amounts occurring in kidney, liver and other organs. In liver and blood plasma, the UQ_{red} homologue amounted to 70–80% of the total UQ (UQ_{ox} + UQ_{red} = t-UQ). UQ_{red} was less than 30% of t-UQ in other tissues and blood cells. t-UQ was much higher in leukocytes and platelets in blood than in erythrocytes. In erythrocytes, t-UQ was exclusively located in the cell membranes. UQ_{ox} and UQ_{red} were also found in all subcellular fractions isolated from liver and kidney in about the same ratio as UQ_{red}/t-UQ was present in the whole organ. The levels of UQ_{ox} and UQ_{red} per mg protein in subcellular fractions from liver were highest in mitochondria, with lesser amounts present in plasma membranes, lysosomes, Golgi complex, nuclei, microsomes and cytosol. In the mitochondria, the outer membranes were richer in t-UQ than the inner membranes. In the Golgi complex, the light and intermediate fractions were rich in t-UQ when compared to the heavy fraction. The possible physiological role of UQ_{ox} and UQ_{red} in tissues and subcellular fractions is discussed.
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It is well-known that ubiquinone (UQ) functions as one of the indispensable electron carriers in the mitochondrial respiratory chain (1). This is the only established role of UQ in mammalian tissues. UQ can be synthesized *de novo* at the mitochondrial inner membrane (2) where the respiratory chain is located.

However, UQ has been detected not only in mitochondria, but also in other subcellular fractions (3,4). In addition, some of the UQ in tissues was reported not to be the oxidized form (UQ_{ox}), but the reduced form, ubiquinol (UQ_{red}) (5,6). UQ was also found in human and in animal blood plasma (7,8). A certain level of serum UQ is detected even in patients on long-term UQ-free total parenteral nutrition (9). Therefore, UQ appears to be a native component of plasma lipids.

Recently, Dallner and colleagues (4,10) showed that subcellular organelles of rat liver other than the mitochondria, such as the endoplasmic reticulum and Golgi apparatus, are able to synthesize UQ *de novo* and that UQ synthesized at these sites was then secreted into the bloodstream by the endoplasmic reticulum/Golgi system.

In a previous paper (11) we reported that 85% of the total amount of UQ_{ox} and UQ_{red} (t-UQ) in serum exists as UQ_{red}, and that such a high ratio of UQ_{red}/t-UQ was maintained even when the level of t-UQ in serum was enhanced by oral supplementation with UQ_{ox}. This means that UQ_{ox} taken orally is also reduced to UQ_{red} at the expense of reducing equivalents such as NAD(P)H. It is not known why serum needs to maintain such a high level of UQ_{red} and where and how UQ_{ox}, either taken orally or synthesized *de novo*, is hydrogenated and converted to UQ_{red}.

However, these observations strongly suggested that UQ_{ox} and UQ_{red} in plasma and in subcellular fractions other than the mitochondria may play some physiologically important roles. Therefore, a systematic analysis of the distribution of UQ_{ox} and UQ_{red} in animal tissues and subcellular organelles was considered necessary.

In this study we have analyzed the amounts of t-UQ and UQ_{red} homologues in subcellular fractions of liver and kidney, as well as other rat tissues.

MATERIALS AND METHODS

Materials. The UQ_{ox}-9 and UQ_{ox}-10 were donated by Eisai Co. (Bunkyo-ku, Tokyo, Japan). UQ_{red} homologues were prepared by reducing the corresponding UQ_{ox} homologues with 0.25% sodium borohydride in ethanol solution. All other chemicals used were of the highest grade commercially available. Specific pathogen-free, male Wistar rats (8-weeks-old and 180–200 g body weight) were purchased from SLC Co. (Shizuoka, Japan) and fed MF, a commercial diet (Oriental Yeast Co., Tokyo, Japan), prior to the experiments.

Cell fractionation. The rats were anesthetized with diethyl ether, injected with 0.2 mL of a heparin (1,000 U/mL) through the femoral vein, and then dissected. First, blood was drawn from the abdominal aorta into a heparinized vacutainer. Next, the liver was perfused with 20 mL of a chilled isotonic saline through the hepatic portal vein to the inferior *vena cava*, and then the organs intended for UQ analysis were removed. Blood was fractionated into plasma and cells, such as erythrocytes, leukocytes and platelets, using the Shibata and Kobayashi method (12). The erythrocyte fraction was purified by removing leukocytes and platelets by the Beutler and West method (13). Ghost cells and endosomes of erythrocytes were prepared by the method of Dodge *et al.* (14). Upon removal, the organs were homogenized immediately with 4 vol of chilled 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid using a Potter-Elvehjem glass-Teflon homogenizer. Nuclei, mitochondria, crude lysosomes, crude microsomes (containing Golgi complex) and the cytosolic fraction were prepared from tissue homogenates by differential centrifugation at 600 × *g* for 10 min,

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Abbreviations: LDH, lactate dehydrogenase; LDL, low-density lipoprotein; Mg^{2+}-ATPase, Na^+,K^+-insensitive Mg^{2+}-adenosinetriphosphatase; Na^+-K^+-ATPase, Na^+,K^+-sensitive Mg^{2+}-adenosinetriphosphatase; NADPH-cyt.c, NADPH-cytochrome c; RNA, ribonucleic acid; suc-cyt.c, succinate-cytochrome c; UQ, ubiquinone; UQ_{ox}, oxidized form of ubiquinone; UQ_{red}, reduced form of ubiquinone (ubiquinol); t-UQ, oxidized and reduced forms of ubiquinone.

6,500 × *g* for 20 min, 10,000 × *g* for 15 min, 105,000 × *g* for 60 min and 105,000 × *g* for 60 min, respectively, essentially as described by Hogeboom (15). The nuclear fraction was purified further by the Blobel and Potter method (16) from the residues precipitated at the centrifugation step at 600 × *g* for 10 min. The purities of the subcellular fractions were ascertained by measuring marker enzyme activities. The distribution of marker enzymes in the subcellular fractions prepared from liver and kidney is summarized in Table 1.

Nucleoli, nucleolar wash and extranucleolar fractions were isolated from the nuclear fraction by the Muramatsu and Busch method (17).

Subfractions of the mitochondrial inner and outer membranes and the matrix were prepared from the mitochondrial pellets (see above) by the method of Sottocasa *et al.* (18). The specific activities (means ± SEM of three preparations, nmol/mg protein/min) of succinate-cytochrome c (suc-cyt.c) reductase, a marker enzyme of the inner membranes, were 229 ± 61 for the inner membrane fractions, 42.7 ± 18.2 (19% that of the inner membranes) for the outer membrane fractions, and 5.2 ± 4.4 (2%) for the matrix fractions. On the other hand, the specific activities (means ± SEM of three preparations, nmol/mg protein/min) of monoamine oxidase (EC 1.4.3.4), a marker enzyme of the outer membranes, were 105 ± 12 for the outer membrane fractions, 10.4 ± 5.3 (10% of that of the outer membranes) for the inner membrane fractions, and 10.6 ± 2.5 (10%) for the matrix fractions.

The crude lysosomes were further fractionated to the following subfractions: Ly-1 ($d \leqslant 1.109$), Ly-2 ($1.109 < d < 1.135$), Ly-3 ($1.135 < d < 1.145$) and Ly-4 ($1.145 \leqslant d$), using discontinuous metrizamide gradient centrifugation as described by Wattiaux *et al.* (19). From the marker enzyme activities, 70% of lysosomes in the crude lysosomes was located in Ly-2, which also contained 16% of mitochondria, 41% of microsomes and 3% of peroxisomes found in the crude lysosomes. At the same time, 76% of mitochondria and 83% of peroxisomes in the crude lysosomes were found in Ly-4.

Subfractions of the Golgi complex, *i.e.,* the light fraction, the intermediate fraction and the heavy fraction, were prepared directly from the liver homogenates of rats which had been orally given 0.6 g ethanol/100 g body weight (as 50% ethanol solution) 90 min before the enucleation using the sucrose density-gradient centrifugation method of Ehrenreich *et al.* (20). Contamination of the Golgi fractions with other subcellular fractions were estimated to be less than 3% based on measurement of marker enzyme activities, *i.e.,* suc-cyt.c reductase, acid phosphatase (EC 3.1.3.2),, NADPH-cytochrome c (NADPH-cyt.c) reductase (EC 1.6.2.4) and lactate dehydrogenase (LDH, EC 1.1.1.27), and ribonucleic acid (RNA) content.

Smooth and rough endoplasmic reticular fractions were also separated by the Ikehara and Pitot method (21) from the residues obtained by centrifugation at 75,000 × *g* for 3 h in preparing the Golgi fractions mentioned previously. RNA levels (means ± SEM of three preparations, nmol/mg protein), a marker of the rough endoplasmic reticulum, were 201 ± 4 for the rough endoplasmic reticular fractions and 22 ± 6 (11% of the former) for the smooth endoplasmic reticular fractions. Contamination of the microsomal fractions with other subcellular fractions were estimated to be less than 5%, based on measurements of marker enzyme activities.

Plasma membrane fractions, independent of previously described sequential fractionation of subcellular fractions, were also prepared from normal rat liver by the method of Koizumi *et al.* (22). The specific activities of the marker enzymes in the plasma membranes are also shown in Table 1.

Enzyme assays. Microsomal NADPH-cyt.c reductase activity was determined based on the rate of reduction of ferricytochrome c by the subcellular fractions in the presence of NADPH (23). The activity of suc-cyt.c

TABLE 1

Distribution of Marker Enzyme Activities in Subcellular Fractions of Rat Liver and Kidney[a]

Subcellular fractions	Suc-cyt.c reductase	Acid phosphatase	NADPH-cyt.c reductase	LDH	Mg^{2+}-ATPase	Na^{+}-K^{+}-ATPase
Liver						
Nuclei	7.1 ± 4.7	1.4 ± 0.2	11.8 ± 3.9	2.1 ± 1.4	5.9 ± 2.1	4.1 ± 4.3
Mitochondria	100	9.0 ± 1.5	33.3 ± 17.6	2.8 ± 2.4	15.3 ± 8.0	4.8 ± 6.5
Crude lysosomes	6.4 ± 3.4	100	29.7 ± 8.0	2.1 ± 1.4	11.1 ± 0.4	9.7 ± 4.2
Crude microsomes	1.2 ± 0.7	4.2 ± 3.6	100	1.4 ± 0.7	3.5 ± 1.4	0.3 ± 0.4
Cytosol	1.3 ± 0.9	1.9 ± 1.2	6.0 ± 2.2	100	1.0 ± 0.3	5.6 ± 7.9
Plasma membranes	9.2 ± 1.6	4.3 ± 2.0	25.4 ± 1.9	0.7 ± 0.0	100	100
Kidney						
Nuclei	34.0 ± 13.7	16.6 ± 4.7	6.9 ± 4.9	5.4 ± 2.9	—	—
Mitochondria	100	26.7 ± 1.6	12.7 ± 6.2	4.0 ± 2.2	—	—
Crude lysosomes	15.6 ± 7.2	100	36.1 ± 18.4	2.6 ± 1.7	—	—
Crude microsomes	8.5 ± 2.7	9.9 ± 2.3	100	6.4 ± 3.3	—	—
Cytosol	3.7 ± 2.6	3.3 ± 3.0	11.8 ± 5.5	100	—	—

[a]The values are means ± SEM of relative enzyme activities (%) measured on four rats used for liver preparations and three rats used for kidney preparations. The specific activities of marker enzymes in liver and kidney were as follows (respectively): 157 ± 22 and 22.8 ± 3.6 nmol/mg protein/min for succinate-cytochrome c (suc-cyt.c) reductase in the mitochondria; 745 ± 26 and 12.8 ± 3.3 nmol/mg protein/min for acid phosphatase in crude lysosomes; 51 ± 5 and 13.3 ± 0.6 nmol/mg protein/min for NADPH-cyt.c reductase in the crude microsomes; and 14.1 ± 0.1 and 6.72 ± 3.23 units/mg protein/min for lactate dehydrogenase (LDH) in cytosol. The specific activities of Na^{+},K^{+}-insensitive Mg^{2+}-adenosinetriphosphatase (Mg^{2+}-ATPase) and Na^{+},K^{+}-sensitive Mg^{2+}-adenosinetriphosphatase (Na^{+}-K^{+}-ATPase) in plasma membranes from rat liver were 28.7 ± 1.7 and 4.10 ± 0.65 μmol phosphate released/mg protein/h, respectively. These values were defined as 100% of relative enzyme activity for each marker enzyme.

reductase was determined by the Tisdale method (24). The activity of acid phosphatase, a marker enzyme for lysosomes, was measured by the Walter and Schütt method (25), and LDH activity, a marker enzyme for the cytosol, was measured by the Bergmeyer and Bernt method (26). The activities of Na^+-K^+-insensitive and Na^+-K^+-sensitive Mg^{2+}-adenosinetriphosphatases (Mg^{2+}-ATPase and Na^+-K^+-ATPase, EC 3.6.1.3), which are marker enzymes for plasma membranes, were measured by the Komatsu and Fujii method (27). Monoamine oxidase activity was assayed by the Nagatsu method (28). The activities of catalase (EC 1.11.1.6), a marker enzyme for peroxisomes, was measured by the method of Baudhuin *et al.* (29). RNA content was assayed by the Le Pecq and Paoletti method (30), and the protein content of subcellular fractions by the method of Lowry *et al.* (31).

Determination of UQ_{red} and t-UQ. The UQ_{ox} and UQ_{red} homologues in the tissues and subcellular fractions were extracted with ethanol and *n*-hexane in a nitrogen atmosphere and determined by high-performance liquid chromatography with electrochemical detection, as previously described (32).

RESULTS

Distribution of UQ_{ox} and UQ_{red} homologues in rat tissues. The content of t-UQ and UQ_{red} homologues in plasma and some major organs of Wistar rats is summarized in Table 2. Two kinds of UQ homologues of UQ-9 and UQ-10 were detected in the tissues analyzed. UQ-9 was the predominant homologue and UQ-10 was a minor one in all of the tissues sampled. The amounts of UQ_{red}-9 and UQ_{red}-10 were 70–80% of the corresponding t-UQ homologues in liver and plasma, but were less than 30% in heart, kidney and spleen. Low amounts of the UQ_{red} forms were also observed in lung, brain and skeletal muscle (data not shown). The difference between the amounts of UQ_{red} in the liver and in other organs could not be due to the oxidation of UQ_{red} to UQ_{ox} during extraction because all organs were treated equally. The samples were always prepared from freshly removed organs, and the UQ_{red} forms were measured immediately to avert oxidation to the corresponding UQ_{ox} forms.

Distribution of UQ_{ox} and UQ_{red} homologues in rat blood cells. The content of t-UQ and UQ_{red} homologues in rat blood cells is summarized in Table 3. UQ-9 and UQ-10 were detected in all blood cells isolated. Leukocytes and platelets, which have mitochondria, contained higher amounts of t-UQ-9 and t-UQ-10 than did erythrocytes, which do not have mitochondria. The UQ_{red} forms were below 10% of the t-UQ in erythrocytes and leukocytes, although they were not detected in platelets. In erythrocytes, interestingly, UQ was detected only in the cell membranes (ghost cells). This may be due to the lack of a biosynthetic system for UQ in erythrocytes.

Subcellular distribution of UQ_{ox} and UQ_{red} homologues in rat liver and kidney. The levels of t-UQ and UQ_{red} homologues in subcellular fractions prepared from fresh rat liver and kidney are listed in Tables 4 and 5, respectively. All fractions tested contained significant amounts of UQ-9 and UQ-10. The levels of the UQ_{red} forms reached 60–70% of those of the t-UQ homologues in most subcellular fractions of liver and amounted to about 25% of those in kidney. The content of the t-UQ homologues in plasma membranes was comparable to that in mitochondria, but the amounts of the UQ_{red} forms present were half of those in the latter fractions. These extremely low levels of the UQ_{red} forms in plasma membranes may be partly due to their oxidation to the corresponding UQ_{ox} forms during the rather complicated isolation process. On the basis of protein content, the crude microsomes, a mixture of both the endoplasmic reticulum and Golgi complex, which may be involved in the *de novo* synthesis of UQ and in supplying UQ for blood plasma (4), showed the lowest level of t-UQ homologues among the subcellular organelles. The t-UQ content in the crude lysosomes from liver and kidney, which are known to efficiently take up extracellular UQ (33) was, respectively, about one-fourth and one-half of that in mitochondria. However, these low levels of t-UQ in crude lysosomes from liver appeared to be contaminated with microsomes and peroxisomes, because the levels in lysosomes, which were purified by metrizamide-gradient centrifugation, were almost equal to those in mitochondria. The cytosolic fractions obtained at the final stage of the cell fractionation process tended to show higher levels of UQ_{red} forms

TABLE 2

Distribution of Ubiquinone (UQ) and Ubiquinol Homologues in Rat Tissues[a]

Tissues	Rats (n)	UQ homologues detected	t-UQ[b] content	UQ_{red} content	UQ_{red} (% of t-UQ)[c]
Plasma	8	UQ-9	0.48 ± 0.01	0.39 ± 0.02	82.1 ± 7.3
		UQ-10	0.12 ± 0.01	0.09 ± 0.01	75.9 ± 8.5
Liver	7	UQ-9	105.1 ± 2.3	78.0 ± 3.8	74.5 ± 10.5
		UQ-10	18.8 ± 1.1	12.7 ± 1.0	67.4 ± 5.1
Heart	5	UQ-9	188.4 ± 5.8	17.4 ± 0.4	9.3 ± 0.7
		UQ-10	21.6 ± 0.6	1.88 ± 0.05	8.8 ± 0.8
Kidney	4	UQ-9	122.6 ± 3.6	19.9 ± 1.9	16.2 ± 2.8
		UQ-10	31.8 ± 0.9	4.89 ± 0.09	15.3 ± 2.8
Spleen	4	UQ-9	75.9 ± 3.2	22.4 ± 0.8	29.8 ± 3.9
		UQ-10	39.6 ± 1.0	9.77 ± 0.23	24.7 ± 0.7

[a]The values are means ± SEM (μg/g wet tissues or mL plasma) of the number of rats listed.
[b]The sum of oxidized form of UQ (UQ_{ox}) and reduced form of UQ (ubiquinol) (UQ_{red}) of each of the homologues.
[c]{[UQ_{red}]/[t-UQ]} × 100.

TABLE 3

Distribution of Ubiquinone and Ubiquinol Homologues in Blood Cells[a]

Fractions	UQ homologues detected	t-UQ content[b] (ng/mg protein)	UQ_{red} content (ng/mg protein)	UQ_{red} (% of t-UQ)[c]
Plasma	UQ-9	0.99 ± 0.57	0.68 ± 0.28	80.2 ± 5.1
	UQ-10	0.19 ± 0.10	0.15 ± 0.07	76.5 ± 0.9
Erythrocytes	UQ-9	0.13 ± 0.02	0.01 ± 0.01	8.3 ± 4.4
	UQ-10	0.06 ± 0.02	0.01 ± 0.00	9.2 ± 4.4
Ghost cells	UQ-9	12.51 ± 3.97	0.31 ± 0.10	2.5 ± 0.3
	UQ-10	4.23 ± 2.49	0.02 ± 0.03	0.6 ± 0.8
Endosomes	UQ-9	0	0	—
	UQ-10	0	0	—
Leukocytes	UQ-9	42.10 ± 8.85	1.18 ± 1.66	3.6 ± 5.0
	UQ-10	20.37 ± 6.80	0.89 ± 0.65	5.6 ± 4.0
Platelets	UQ-9	40.02 ± 6.34	n.d.	—
	UQ-10	30.59 ± 17.25	n.d.	—

[a]The blood samples were mixed with 0.075 vol of 0.077 mM EDTA (pH 7.4), centrifuged at 250 × *g* for 10 min to separate erythrocytes and leukocytes, and then at 800 × *g* for 15 min to separate platelets. The values are means ± SEM of three rats; n.d., not detected; other abbreviations as in Table 2.
[b]The sum of UQ_{ox} and UQ_{red} forms of each of the homologues.
[c]$\{[UQ_{red}]/[t\text{-}UQ]\} \times 100$.

TABLE 4

Distribution of Ubiquinone and Ubiquinol Homologues in Rat Liver Subcellular Fractions[a]

Subcellular fractions	Rats (n)	UQ homologues detected	t-UQ content[b] (ng/mg protein)	UQ_{red} content (ng/mg protein)	UQ_{red} (% of t-UQ)[c]
Homogenates	7	UQ-9	279 ± 25	224 ± 22	80 ± 3
		UQ-10	31.0 ± 6.9	24.1 ± 7.0	80 ± 7
Nuclei	11	UQ-9	322 ± 104	219 ± 66	67 ± 6
		UQ-10	31.7 ± 10.8	22.2 ± 7.8	69 ± 5
Mitochondria	11	UQ-9	1,780 ± 370	1,280 ± 270	73 ± 7
		UQ-10	218 ± 63	162 ± 51	74 ± 7
Crude lysosomes	11	UQ-9	447 ± 80	308 ± 64	67 ± 10
		UQ-10	83.3 ± 23.3	51.5 ± 11.5	63 ± 9
Crude microsomes	11	UQ-9	121 ± 41	83 ± 31	69 ± 7
		UQ-10	17.7 ± 3.4	11.8 ± 3.0	66 ± 9
Cytosol	11	UQ-9	21.3 ± 5.7	16.9 ± 5.3	74 ± 7
		UQ-10	2.37 ± 1.11	1.53 ± 0.58	72 ± 5
Plasma membranes	4	UQ-9	1,550 ± 170	510 ± 100	33 ± 3
		UQ-10	235 ± 79	71 ± 28	30 ± 7

[a]The values are means ± SEM of the number of rats listed. Abbreviations as in Table 2.
[b]The sum of UQ_{ox} and UQ_{red} forms of each of the homologues.
[c]$\{[UQ_{red}]/[t\text{-}UQ]\} \times 100$.

TABLE 5

Distribution of Ubiquinone and Ubiquinol Homologues in Rat Kidney Subcellular Fractions[a]

Subcellular fractions	UQ homologues detected	t-UQ[b] content (ng/mg protein)	UQ_{red} content (ng/mg protein)	UQ_{red} (% of t-UQ)[c]
Nuclei	UQ-9	700 ± 171	124 ± 48	23 ± 2
	UQ-10	83.5 ± 59.8	24.4 ± 18.8	24 ± 4
Mitochondria	UQ-9	901 ± 89	232 ± 19	26 ± 1
	UQ-10	98.3 ± 53.5	25.3 ± 12.8	27 ± 2
Crude lysosomes	UQ-9	478 ± 301	150 ± 74	25 ± 3
	UQ-10	69.0 ± 7.9	16.1 ± 1.4	24 ± 3
Crude microsomes	UQ-9	197 ± 113	58 ± 28	24 ± 1
	UQ-10	88.3 ± 54.7	22.2 ± 14.0	25 ± 3
Cytosol	UQ-9	52.1 ± 38.9	15.2 ± 7.7	27 ± 3
	UQ-10	5.20 ± 0.94	1.45 ± 0.41	27 ± 3

[a]The values are means ± SEM of three rats. Abbreviations as in Table 2.
[b]The sum of UQ_{ox} and UQ_{red} forms of each of the homologues.
[c]$\{[UQ_{red}]/[t\text{-}UQ]\} \times 100$.

than did other subfractions. This may be attributed to a UQ reductase activity in the cytosol, which can reduce UQ_{ox} to UQ_{red} in the presence of NADPH (23).

Liver nuclei were fractionated further into three fractions, namely nucleoli, a nucleolar wash and an extranucleolar fraction. Liver mitochondria were also separated into three fractions, inner membranes, outer membranes and matrix. The distribution of t-UQ-9 and t-UQ-10 in these fractions is shown in Table 6. The amounts of UQ_{red} in the mitochondrial and nuclear fractions were excluded from the table, as they had decreased to about 50–70% of the original levels of UQ_{red}. This was probably due to autoxidation during fractionation (data not shown). In mitochondria, surprisingly, the amounts of t-UQ-9 and t-UQ-10 in the outer membranes were about twice as high as those in the inner membranes where UQ participates in the respiratory chain. Furthermore, the matrix fraction also contained a high amount of t-UQ, which is comparable to that in the inner membranes.

The crude lysosomes were further fractionated to subfractions Ly-1 to Ly-4 by discontinuous metrizamide gradient centrifugation as shown in Table 7. Most proteins of the crude lysosomes were located in Ly-2 and Ly-4. Based on marker enzyme activities, Ly-2 was a lysosome-rich fraction, whereas Ly-4 was rich in mitochondria and peroxisomes.

Rough and smooth endoplasmic reticula and Golgi complex were also isolated from the crude microsomal fractions. The distribution of t-UQ-9 and t-UQ-10 in these fractions is shown in Table 8. The amounts of UQ_{red} homologues were omitted from the table because they had dropped to 35–75% of the levels of the original crude microsomes during the preparation. The amounts of t-UQ in Golgi subfractions were 5–6 times higher than those in submicrosomal fractions. Furthermore, the content of t-UQ homologues, in light and in intermediate fractions, was more than three times higher than that in the heavy fraction. This is in line with the hypothesis that UQ, either synthesized *de novo* or taken up by the endoplasmic reticulum, is concentrated at the Golgi complex before exocytosis (4).

TABLE 6

Distribution of Total Ubiquinones in Rat Liver Nuclei and Mitochondria[a]

Fractions	t-UQ-9	t-UQ-10
Nuclei	302 ± 132	34.1 ± 14.6
Nucleoli	518 ± 216	50.1 ± 26.6
Nucleolar wash	609 ± 204	54.0 ± 26.6
Extranucleolar fraction	245 ± 63	32.2 ± 9.1
Mitochondria	1,670 ± 360	225 ± 76
Inner membranes	559 ± 226	125 ± 68
Outer membranes	1,290 ± 380	284 ± 75
Matrix	339 ± 138	86 ± 41

[a]The values are means ± SEM (ng/mg protein) of seven rats. t-UQ is the sum of UQ_{ox} and UQ_{red} of each homologue. Abbreviations as in Table 2.

TABLE 7

Distribution of Total Ubiquinones in Rat Liver Lysosomes[a]

Fractions	Proteins found (%)	Marker enzyme activity (%)[b]		UQ content (ng/mg protein)[c]	
		Acid phosphatase	Catalase	t-UQ-9	t-UQ-10
Crude lysosomes	100	100	100	542 ± 62	86.9 ± 12
Ly-1	3.79 ± 1.96	162 ± 24	2.0 ± 0.6	1,430 ± 260	269 ± 76
Ly-2	20.4 ± 4.9	207 ± 50	5.9 ± 2.2	1,560 ± 350	292 ± 80
Ly-3	8.56 ± 2.06	88.7 ± 10.1	70.6 ± 11.7	598 ± 75	103 ± 15
Ly-4	18.5 ± 3.2	18.6 ± 6.1	188 ± 24	447 ± 86	42.3 ± 5.8

[a]The values are means ± SEM of four rats. Abbreviations as in Table 2.
[b]The specific activities of acid phosphatase and catalase in crude lysosomes were 791 ± 118 nmol/mg protein/min and 23.3 ± 6.3 units/mg protein, respectively.
[c]t-UQ is the sum of UQ_{ox} and UQ_{red} of each homologue.

TABLE 8

Distribution of Total Ubiquinones in Rat Liver Microsomes and Golgi Complex[a]

Fractions	t-UQ-9	t-UQ-10
Crude microsomes	122 ± 50	17.3 ± 3.3
Smooth endoplasmic reticulum	144 ± 64	23.6 ± 10.7
Rough endoplasmic reticulum	119 ± 43	26.5 ± 20.1
Golgi complex		
Light fraction	676 ± 232	252 ± 141
Intermediate fraction	723 ± 539	260 ± 160
Heavy fraction	212 ± 126	94.4 ± 48.7

[a]The values are means ± SEM (ng/mg protein) of seven rats. t-UQ is the sum of UQ_{ox} and UQ_{red} of each homologue. Abbreviations as in Table 2.

DISCUSSION

The present results show that all tissues and subcellular fractions isolated from both liver and kidney contain significant amounts of UQ, as was reported by Kalén *et al.* (4,34). The data also reveal that 70–80% of the total amounts of each UQ_{ox} and UQ_{red} homologue in the liver and plasma, as well as 20–30% of those in other tissues, exist as the reduced hydroquinone form. Recently, Matsura *et al.* (35) suggested, based on studies in rabbits, that each UQ homologue may have a specific function with UQ-10 acting as an electron carrier and UQ_{red}-9 as an antioxidant. In rats, however, the distribution patterns of UQ-9 and UQ-10 were not that different between mitochondria and other subcellular fractions.

Recently, Dallner and colleagues (10) reported that nonaprenyl-4-hydroxybenzoate transferase, an enzyme responsible for UQ biosynthesis, was detected in all subcellular fractions except for peroxisomes and cytosol from rat liver. Kang *et al.* (36) also found 1-methoxy-5-polyprenylphenol, a known intermediate of UQ synthesis, in a microsomal fraction from human hepatoma cells, though they could not detect the activity of *p*-hydroxybenzoate polyprenyl transferase in this fraction. These findings suggest that most subcellular organelles, including mitochondria, have their own pathways of UQ biosynthesis. These findings, as well as our results, further indicate that UQ may be one of the intrinsic structural components of subcellular membranes, just like cholesterol or α-tocopherol, and that UQ_{red} may play an important role in membrane physiology.

It is still obscure whether the UQ_{ox} and/or the UQ_{red} present in subcellular fractions other than mitochondria serve some indispensable function. It has been suggested that UQ_{red} may act as a lipid-soluble antioxidant (37). According to Kagan *et al.* (38,39), however, UQ_{red} is a much weaker antioxidant against lipid peroxidation than is α-tocopherol, and the antioxidative effect of long chain homologues, such as UQ_{red}-9 and UQ_{red}-10, is much weaker than that of short chain homologues, shorter than UQ_{red}-5. Therefore, it is still controversial whether or not the long chain homologues existing widely in animal tissues serve as physiological antioxidant systems. However, some disagreements on the antioxidant effects of UQ_{red} appear to result from a disregard of the location of UQ homologues in bilayer lipid membranes. It has been assumed that the long chain UQ homologues, such as UQ-10, are mainly located in the hydrophobic core of the bilayer, while the short chain homologues are assumed to be located near the membrane surface (40,41) in parallel with aliphatic chains of the phospholipids with their polar heads exposed beyond the membrane surface, similar to α-tocopherol. Therefore, the antioxidative effects of native UQ homologues, such as UQ_{red}-9 and UQ_{red}-10, should be examined under experimental conditions in which the molecules are appropriately located, *i.e.*, within the hydrophobic core of the membranes. Leibovitz *et al.* (42) showed that lipid peroxidation induced by *t*-butyl hydroperoxide in tissue slices from rats was depressed by feeding the rats diets fortified with UQ_{ox}-10 before the experiment. Marubayashi *et al.* (43) reported that in hepatic ischemia and subsequent reperfusion, the decrease in the levels of the reduced form of glutathione was not observed in rats pretreated by UQ_{ox}-10 injections. Frei *et al.* (44) reported that UQ_{red}-10, but not UQ_{ox}-10, acted as an effective antioxidant against lipid peroxidation induced by 2,2′-azo*bis*(2,4-dimethyl-valeronitrile) in lecithin liposomes and that UQ_{red}-10 could work in concert with α-tocopherol through a coupled redox cycling system. According to these findings, the UQ_{red} forms appear to be more important antioxidants than are the UQ_{ox} forms. The fact that about one-fourth of t-UQ exists in the hydroquinone form, which seems to be formed by consuming reducing equivalents equimolar to NAD(P)H (23), supports the concept under consideration.

UQ in plasma is mostly associated with lipoprotein fractions (8); plasma itself does not seem to have UQ-reducing activity (data not shown). Extremely high levels of UQ_{red} in both liver and plasma, in comparison with other tissues, lead us to the conclusion as was proposed by Kalén *et al.* (4), that UQ is synthesized and concentrated in the endoplasmic reticulum-Golgi complex system of the liver and then secreted into plasma as lipoprotein vesicles. The high ratio of UQ_{red}/t-UQ in plasma compared to the ratio of UQ_{red}/t-UQ in other tissues is also very interesting. It can be argued that the peroxidation of plasma lipoprotein lipids is one of the causative events in arteriosclerosis (45,46). Recently, Stocker *et al.* (47) reported that UQ_{red}-10 protects human low-density lipoprotein (LDL) more efficiently from lipid peroxidation than does α-tocopherol. The protection of plasma LDL from the peroxidation may thus be a major role of UQ_{red}-10 in plasma. Of course, plasma UQ may also serve as UQ pool for other tissues with insufficient UQ biosynthetic capacity. We recently also found that serum UQ_{ox} is necessary for the regular and rhythmic beating of cultured mouse fetal myocardial cells (48).

Other possible roles of UQ, as Sun *et al.* (49) reported, may include that UQ could serve as a transmembrane electron carrier in plasma membrane redox systems and may be essential for cell growth. In addition, it has also been assumed that UQ may contribute to the fluidity of phospholipid bilayer membranes (50) and may control fatty acid release from membrane phospholipids by phospholipases (51).

High levels of UQ_{red} must be maintained by consuming biological reducing equivalents, such as NAD(P)H, in the presence of the proper enzyme systems. Takada *et al.* (52) reported that in guinea pig liver, microsomes and cytosol, as well as mitochondria, had a reducing activity for UQ_{ox}-10 and that DT-diaphorase (= quinone reductase, EC 1.6.99.2) was one of the enzymes responsible for the reduction of UQ_{ox}-10 in cytosol. DT-diaphorase is widely distributed in animal tissues, but its favorite substrates are not the long chain UQ_{ox} homologues, but rather vitamin K and the short chain UQ_{ox} homologues (53). We recently found that in the liver cytosol there exists a novel NADPH-UQ reductase activity, a favorite substrate of which is UQ_{ox} rather than naphthoquinone derivatives (23). Therefore the question still remains to be answered: Whether or not the high levels of UQ_{red} in tissues, especially in the liver, can be maintained by DT-diaphorase or NADPH-UQ reductase, or both, possibly in cooperation with some other enzymes. Further studies are necessary to elucidate the mechanisms of the reduction of UQ_{ox}, as well as its physiological role at sites other than mitochondria.

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Effect of Dietary α-Linolenic Acid and Its Ratio to Linoleic Acid on Platelet and Plasma Fatty Acids and Thrombogenesis

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The effect of dietary α-linolenic acid (18:3n-3) and its ratio to linoleic acid (18:2n-6) on platelet and plasma phospholipid (PL) fatty acid patterns and prostanoid production were studied in normolipidemic men. The study consisted of two 42-d phases. Each was divided into a 6-d pre-experimental period, during which a mixed fat diet was fed, and two 18-d experimental periods, during which a mixture of sunflower and olive oil [low 18:3n-3 content, high 18:2/18:3 ratio (LO-HI diet)], soybean oil (intermediate 18:3n-3 content, intermediate 18:2/18:3 ratio), canola oil (intermediate 18:3n-3 content, low 18:2/18:3 ratio) and a mixture of sunflower, olive and flax oil [high 18:3n-3 content, low 18:2/18:3 ratio (HI-LO diet)] provided 77% of the fat (26% of the energy) in the diet. The 18:3n-3 content and the 18:2/18:3 ratio of the experimental diets were: 0.8%, 27.4; 6.5%, 6.9; 6.6%, 3.0; and 13.4%, 2.7, respectively. There were appreciable differences in the fatty acid composition of platelet and plasma PLs. Nevertheless, 18:1n-9, 18:2n-6 and 18:3n-3 levels in PL reflected the fatty acid composition of the diets, although very little 18:3n-3 was incorporated into PL. Both the level of 18:3n-3 in the diet and the 18:2/18:3 ratio were important in influencing the levels of longer chain n-3 fatty acid, especially 20:5n-3, in platelet and plasma PL. Production of 6-keto-$PGF_{1\alpha}$ was significantly ($P < 0.05$) higher following the HI-LO diet than the LO-HI diet although dietary fat source had no effect on bleeding time or thromboxane B_2 production. The present study showed that both the level of 18:3n-3 in the diet and its ratio to 18:2n-6 were important in influencing long-chain n-3 fatty acid levels in platelet and plasma PL and that prostanoid production coincided with the diet-induced differences in PL fatty acid patterns.

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Coronary heart disease is a degenerative process involving atherosclerosis and thrombogenesis (1,2). Dietary fat affects both of these phenomena (3). Polyunsaturated fatty acids (PUFA) of the n-3 and n-6 families may influence thrombus formation through their effects on the production of prostanoids in the endothelial wall and in the circulating platelets (4-6).

Dietary linoleic acid (18:2n-6) can be desaturated and elongated to arachidonic acid (20:4n-6), which is the precursor of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) (6,7). Similarly, dietary α-linolenic acid (18:3n-3) can be desaturated and elongated to eicosapentaenoic acid (20:5n-3), the precursor of TXA_3 and PGI_3 (6,7), but the efficiency of conversion has been reported to be relatively low in humans (8,9). TXA_2 is a potent prothrombogenic agent whereas TXA_3 is a weak platelet aggregating agent. By contrast, both PGI_2 and PGI_3 are antithrombogenic (2).

Most of the research on the antithrombogenic effect of dietary fatty acids has focused on dietary 20:5n-3, mainly because it is the direct precursor of antithrombogenic prostanoids and a powerful inhibitor of n-6 PUFA metabolism (9). However, 20:5n-3 and docosahexaenoic acid (22:6n-3) are not effective in lowering blood cholesterol levels (10). Thus, interest has turned to the possible antiatherogenic and antithrombogenic role of dietary 18:3n-3. Both 18:2n-6 and 18:3n-3 share the same enzyme system for desaturation and elongation (2,6,7); hence, there is the possibility for competition between them. Thus, the relative and absolute amounts of these fatty acids in the diet could have an effect on the metabolism of n-6 and n-3 fatty acids which, in turn, could alter prostanoid metabolism and thrombogenesis. The importance of the 18:2/18:3 ratio in the diet has been studied by several investigators (8,11,12). However, a comparison of the effect of the absolute amounts of 18:2n-6 and 18:3n-3 and their ratio in the diet on plasma fatty acid patterns and thrombogenic tendency in the human has not been reported.

The present study was designed to investigate the effect of dietary 18:2n-6 and 18:3n-3 and their ratio on n-3 and n-6 PUFA patterns in platelet and plasma phospholipids (PL) and on bleeding time and on bleeding-time prostanoid formation. Fatty acid patterns of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and alkenylacyl glycerophosphoethanolamines (PPE) are reported. PPE has been shown to be the major storage site for 20:5n-3, although an appreciable amount also is found in the diacyl PC and PE fractions of platelet PL (13,14).

MATERIALS AND METHODS

Experimental design. The study consisted of two 42-d phases with a 2-mon break between phases. Each phase was divided into three periods: a 6-d pre-experimental period and two 18-d experimental periods. Eight male subjects participated in the study. All subjects were fed a mixed fat diet during the pre-experimental period of each phase. In the first experimental period of phase I, two subjects were randomly assigned to one of four experimental diets: sunflower and olive oil [low 18:3n-3 content, high 18:2/18:3 ratio (LO-HI)]; soybean oil [intermediate 18:3n-3 content, intermediate 18:2/18:3 ratio (IN-IN)]; canola oil [intermediate 18:3n-3 content, low 18:2/18:3 ratio (IN-LO)]; and sunflower, olive and flax oil [high 18:3n-3 content, low 18:2/18:3 ratio (HI-LO)]. In the

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; GLC, gas-liquid chromatography; HI-LO, high 18:3n-3 content, low 18:2/18:3 ratio; IN-IN, intermediate 18:3n-3 content, intermediate 18:2/18:3 ratio; IN-LO, intermediate 18:3n-3 content, low 18:2/18:3 ratio; LO-HI, low 18:3n-3 content, high 18:2/18:3 ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGI, prostacyclin; PL, phospholipid(s); PPE, alkenylacyl glycerophosphoethanolamines; PPP, platelet-poor plasma; PUFA, polyunsaturated fatty acids; TXA, thromboxane.

subsequent experimental periods of phases I and II, each pair of subjects was switched to a different diet according to the following order: LO-HI to IN-IN; IN-IN to IN-LO; IN-LO to HI-LO; and HI-LO to LO-HI.

Subjects. The subjects, who ranged in age from 20 to 34 yr, were students at the University of Manitoba. They were normolipidemic with plasma total cholesterol levels between 3.80 and 5.86 mmol/L at the beginning of the study. The subjects engaged in their normal activities and resided in their own residences throughout the study. They were instructed not to use aspirin during the study and to consult the directors before taking any medication. One subject left after the completion of phase I, and a replacement was recruited for phase II. All meals were served in the metabolic unit located in the Faculty of Human Ecology. The study protocol was approved by the Faculty of Human Ecology Ethics Committee, and written consent was obtained from each subject.

Diets. A two-day cyclic menu of conventional foods was used during the study. Diets used in the study were identical in all respects except sources of added fat which accounted for approximately 26% of total energy (77% of the 120 g/d of total dietary fat). The fatty acid composition of the diets is presented in Table 1. The LO-HI and the IN-LO diets had similar amounts of oleic acid (18:1n-9) and 18:2n-6, but the latter contained about eight times more 18:3n-3. The IN-IN diet was similar in 18:3n-3 content to the IN-LO diet but contained twice the level of 18:2n-6. The levels of 18:2n-6 and 18:3n-3 in the HI-LO diet were approximately twice those in the IN-LO diet, but the 18:2/18:3 ratio was similar to that of the IN-LO diet (3.0 *vs.* 2.7). A one-day composite of each diet was collected during each phase of the study. The composites were homogenized and aliquots lyophilized for chemical analysis. Fat content of the diets was determined by the method of Bligh and Dyer (15). Fatty acid methyl esters were prepared using sodium methoxide in methanol (16), and the fatty acid composition was determined by gas-liquid chromatography (GLC).

TABLE 1

Fatty Acid Composition of the Diets[a]

	Diets[b]				
Fatty acid	Mixed fat	LO-HI	IN-IN	IN-LO	HI-LO
	Percent of total fatty acids				
14:0	4.5	1.5	1.5	1.5	1.6
16:0	23.8	13.0	14.5	9.8	11.7
16:1n-7	1.8	0.8	0.6	0.7	0.6
18:0	12.4	4.7	5.2	3.8	5.6
18:1n-9	38.5	56.0	24.8	54.2	29.6
18:2n-6	12.0	21.9	44.9	19.5	36.0
18:3n-3	1.0	0.8	6.5	6.6	13.4
20:0	0.3	0.4	0.3	0.6	0.3
20:1n-9	0.5	0.4	0.2	1.3	0.1
22:0	trace[c]	0.3	0.3	0.4	0.4
18:2/18:3 Ratio	12.0	27.4	6.9	3.0	2.7

[a]Means of four duplicates (two of each menu).
[b]Source of added fat: Mixed fat, 11% corn oil, 22% lard, 22% tallow, and 22% shortening; low 18:3n-3 content, high 18:2/18:3 ratio (LO-HI), 20% sunflower oil and 80% olive oil; intermediate 18:3n-3 content, intermediate 18:2/18:3 ratio (IN-IN), 100% soybean oil; intermediate 18:3n-3 content, low 18:2/18:3 ratio (IN-LO), 100% canola oil; and high 18:3n-3 content, low 18:2/18:3 ratio (HI-LO), 47% sunflower oil, 20% olive oil and 33% flax oil.
[c]Less than 0.05%.

Fatty acid analysis of platelet and plasma PL. Blood samples were collected from the subjects into ethylenediaminetetraacetic acid (EDTA)-containing tubes following a 12-h overnight fast at the end of each experimental period. The samples were centrifuged at 250 × *g*, 18°C for 15 min to remove the red blood cells. Platelets were separated from the resulting plasma by centrifuging at 1400 × *g*, 4°C for 15 min. Platelet-poor plasma (PPP) was removed from the samples and stored under nitrogen at −10°C. Washed suspensions of the platelets were prepared by the method of McKean *et al.* (17). The final suspension was in 1 mL of cold buffer (2 mmol Na_2EDTA/L, 0.15 mol NaCl/L, 0.02 mol Tris hydroxyaminomethane/L; pH 7.4). The tubes containing the final suspensions were flushed with nitrogen, stored at −22°C and then shipped on dry ice to the University of Guelph. The platelet suspensions were thawed on ice and the lipids extracted by the method of Bligh and Dyer (15). The PPP samples were thawed in the refrigerator and the lipids extracted immediately by the method of Folch *et al.* (18). The PL in the platelets and plasma were separated using two-directional thin-layer chromatography on pre-coated plates (Merck silica gel 60 HR, Terochem Laboratories Ltd., Toronto, Canada). The spotted plates were first developed in chloroform/methanol/ammonium hydroxide (65:35:5.5, by vol) and then in a second direction in chloroform/methanol/formic acid (55:25:5, by vol). Following the first run, the plates were dried under nitrogen and placed in a chamber containing HCl fumes for 10 min to hydrolyze the alkenyl group from the PPE fraction (19). Following development, the plates were sprayed with dichlorofluorescein dye, exposed to ammonia fumes and the lipid factors were localized under ultraviolet light. The PC, PE and PPE fractions were scraped into test tubes and transmethylated with 6% sulfuric acid in methanol in the presence of known amounts of monopentadecanoin as internal standard (Nu-Chek-Prep, Elysian, MN) in the case of platelet PL and with sodium methoxide in methanol (16) in the presence of internal standard in the case of the plasma PL. Similar areas of gel were scraped from blank plates and processed as for the samples. The fatty acid methyl esters were separated by GLC as described previously for platelets (20) and plasma (21) PL fractions. The amounts of fatty acids in each sample were adjusted by subtracting the amounts present in the spots scraped from the blank plates, relative to the amount of monopentadecanoin in each.

Bleeding time and prostanoid production. Bleeding time and the bleeding-time production of TXB_2, the stable metabolite of TXA_2, and of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, were measured at the end of each experimental period. Duplicate bleeding times were measured by making incisions 5 mm long and 1 mm deep on the volar surface of the arm using a sterile, disposable, standardized device (Simplate, General Diagnostics, Warner Lambert, Morris-Plains, NJ). A constant pressure was maintained with a sphygmomanometer cuff on the upper arm inflated to 44 mm Hg. Bleeding time was measured with a stopwatch. Blood from the edge of the incision was collected into heparinized microhematocrit tubes containing 10 μL of 1 g indomethacin/L for the determination

of TXB_2 and 6-keto-$PGF_{1\alpha}$. Immediately following collection of the blood, 3000 dpm of [^{3}H]PGE_2 (sp. act., 185 Ci/mmol) was added to each sample in order to correct for recovery of TXB_2 and 6-keto-$PGF_{1\alpha}$. The tubes were centrifuged, the plasma volume measured and the plasma removed for prostanoid measurement. Plasma samples were loaded onto a C_{18} Sepak cartridge (Waters Scientific, Mississauga, Canada). Water (20 mL), 10% (by vol) ethanol (20 mL) and petroleum ether (10 mL) were passed through the cartridges, and the prostanoids were then extracted with 7.5 mL methyl formate. This fraction was evaporated under a stream of nitrogen and the sample reconstituted in 0.5 mL bovine serum albumin in phosphate-buffered saline. The amounts of 6-keto-$PGF_{1\alpha}$, and TXB_2 were assayed by using highly specific anti-6-keto-$PGF_{1\alpha}$ and anti-TXB_2 in an enzyme-linked immunosorbent assay (22,23). Bleeding-time values for prostanoid production refer to blood collected in the first 4 min after incision. TXB_2 is expressed as a concentration (μg/L) whereas 6-keto-$PGF_{1\alpha}$ is expressed in terms of rate of production (pg/min) (23,24).

Statistical analysis. The experimental design of the study was a modified Latin square (25). F tests were performed on the estimated diet effects obtained from regression analysis of the data for plasma PL fatty acids, bleeding times and bleeding-time prostanoid production (25). Analyses were performed using the SAS computer program (1984, 1986 SAS Institute Inc., Cary, NC). Diet effects on the platelet fatty acid composition were determined for adjusted treatment means compared by protected least significant differences (26). All means were adjusted for the withdrawal of a subject after the completion of phase I.

RESULTS

Fatty acid patterns of platelet and plasma PL. Fatty acid patterns of platelet (Tables 2–4) and plasma (Tables 5–7) PL reflected the fatty acid composition of the diets even though there were some major differences in fatty acid composition between the platelet and plasma fractions, *e.g.*, appreciably higher 20:4n-6 and lower 18:2n-6 and 22:6n-3 levels in platelet than plasma PL. The 18:1n-9 levels were significantly higher in all PL fractions following the LO–HI and IN–LO diets, which were appreciably

TABLE 2

Mean Platelet Phosphatidylcholine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
16:0	30.4 ± 3.5[a]	31.1 ± 3.3[a]	31.4 ± 3.3[a]	30.0 ± 3.7[a]
18:0	12.5 ± 1.5[a]	14.2 ± 1.7[b]	12.5 ± 1.4[b]	14.6 ± 1.9[c]
18:1	26.0 ± 0.7[a]	20.6 ± 0.9[b]	26.0 ± 1.5[a]	22.2 ± 1.9[c]
18:2n-6	8.9 ± 1.1[a]	13.1 ± 1.6[b]	8.6 ± 0.9[a]	12.0 ± 1.0[c]
18:3n-3	0.2 ± 0.1[a]	0.3 ± 0.1[b]	0.3 ± 0.1[b]	0.4 ± 0.1[b]
20:3n-6	1.5 ± 0.1[a]	1.4 ± 0.2[a]	1.3 ± 0.2[b]	1.4 ± 0.2[a]
20:4n-6	11.3 ± 1.6[a]	11.2 ± 1.5[a]	10.4 ± 0.7[a]	11.0 ± 1.4[a]
20:5n-3	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.2 ± 0.1[b]	0.2 ± 0.1[b]
22:4n-6	0.8 ± 0.2[a]	0.8 ± 0.2[a]	0.7 ± 0.1[b]	0.7 ± 0.1[b]
22:5n-3	0.4 ± 0.1[a]	0.5 ± 0.1[a,b]	0.5 ± 0.1[b]	0.6 ± 0.1[c]
22:6n-3	0.6 ± 0.1[a]	0.5 ± 0.1[a]	0.5 ± 0.2[a]	0.5 ± 0.2[a]

[a]Values in the same row with the same superscript letter (a–c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.

TABLE 3

Mean Platelet Phosphatidylethanolamine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
16:0	6.4 ± 1.0[a]	6.9 ± 0.8[b]	6.6 ± 1.1[a,b]	6.2 ± 1.2[a]
18:0	27.2 ± 2.4[a]	29.2 ± 2.9[b]	26.9 ± 1.4[a]	30.4 ± 1.7[b]
18:1	17.5 ± 1.0[a]	11.4 ± 1.3[b]	20.2 ± 1.6[c]	12.3 ± 1.3[b]
18:2n-6[b]	4.9 ± 0.7	7.4 ± 1.2	4.9 ± 0.6	6.7 ± 0.3
18:3n-3	0.1 ± 0.0[a]	0.2 ± 0.0[b]	0.2 ± 0.0[c]	0.2 ± 0.0[c]
20:3n-6	1.1 ± 0.2[a,b]	1.0 ± 0.2[c]	1.1 ± 0.3[b]	1.0 ± 0.4[a,c]
20:4n-6	31.8 ± 1.8[a]	32.6 ± 2.0[a]	27.9 ± 1.6[b]	32.9 ± 1.7[a]
20:5n-3	0.2 ± 0.1[a]	0.2 ± 0.0[a]	0.3 ± 0.1[b]	0.4 ± 0.1[b]
22:4n-6	2.2 ± 0.5[a]	2.3 ± 0.3[a]	1.9 ± 0.3[a]	1.8 ± 0.5[a]
22:5n-3	1.4 ± 0.4[a]	1.5 ± 0.2[b]	1.5 ± 0.3[b]	1.8 ± 0.6[c]
22:6n-3	1.6 ± 0.6[a,b]	1.5 ± 0.4[a,c]	1.7 ± 0.4[b]	1.1 ± 0.2[c]

[a]Values in the same row with the same superscript letter (a–c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.
[b]Not subjected to statistical analysis due to treatment covariate interaction.

TABLE 4

Mean Platelet Alkenylacyl Glycerophosphoethanolamine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
18:1	3.6 ± 0.6[a]	2.4 ± 0.7[b]	4.1 ± 0.6[c]	2.7 ± 0.5[b]
18:2n-6[b]	1.9 ± 0.6	2.5 ± 0.7	1.7 ± 0.5	2.3 ± 0.5
20:3n-6	0.7 ± 0.3[a]	0.6 ± 0.2[a]	0.7 ± 0.2[a]	0.7 ± 0.2[a]
20:4n-6	65.7 ± 3.7[a]	65.3 ± 2.4[a]	64.8 ± 3.2[a]	64.2 ± 3.6[a]
20:5n-3	0.5 ± 0.2[a]	0.6 ± 0.1[a]	1.1 ± 0.3[b]	1.2 ± 0.3[b]
22:4n-6	11.9 ± 2.0[a]	12.4 ± 1.7[a]	9.9 ± 1.8[b]	10.4 ± 1.8[b]
22:5n-3	6.1 ± 1.0[a]	7.4 ± 1.5[b]	7.2 ± 1.5[b]	9.6 ± 2.0[c]
22:6n-3	4.0 ± 1.3[a]	3.8 ± 1.0[a]	4.7 ± 1.2[a]	3.8 ± 1.1[a]

[a]Values in the same row with the same superscript letter (a-c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.
[b]Not subjected to statistical analysis due to treatment covariate interaction.

higher in 18:1n-9 content (Table 1), than following the IN-IN and HI-LO diets. Similarly, the 18:2n-6 levels were significantly higher following the IN-IN and HI-LO diets than following the LO-HI and IN-LO diets. Although only small amounts of 18:3n-3 were incorporated into PL, the LO-HI diet led to significantly lower levels of 18:3n-3 than the other diets while the levels following the HI-LO diet were significantly higher for the plasma PL (Tables 5-7). Levels of long-chain PUFA in platelet and plasma PL also were affected by the fatty acid composition of the diets although dietary fatty acid composition had no effect on the 20:4n-6 content, the principal PUFA in the PL fraction, except for the lower level in plasma PC following the HI-LO diet (Table 5) and in platelet PE following the IN-LO diet (Table 3). However, differences in PUFA content among the diet groups were less pronounced than for 18:1n-9, 18:2n-6 and 18:3n-3. In general, the PL tended to be higher in long-chain n-3 PUFA following the HI-LO diet and lower following the LO-HI diet although dietary fat source had little effect on 22:6n-3, the major n-3 fatty acid in the PL fractions. The ratio of 18:2/18:3 in the diet also had an effect on PUFA levels in the PL fractions. The levels of 20:5n-3 were significantly higher following the IN-LO and HI-LO diets than following the LO-HI and IN-IN diets in all fractions for both tissues (Tables 2-7). By contrast, the level of 22:4n-6 in the platelet PPE fraction (Table 4) was significantly lower following the IN-LO and HI-LO diets than the LO-HI and IN-IN diets.

Thrombogenic effect of the experimental diets. No differences in bleeding time and in bleeding-time TXB_2 production were observed among the experimental diets, although levels of TXB_2 tended to be lower following the IN-LO and HI-LO diets than following the LO-HI and IN-IN diets (Table 8). Production of 6-keto-$PGF_{1\alpha}$ in blood collected from the bleeding time wounds was significantly higher following the HI-LO diet than the LO-HI diet. 6-Keto-$PGF_{1\alpha}$ production following the IN-LO and IN-IN diets fell midway between the values on the HI-LO and LO-HI regimen. As a result, the 6-keto-$PGF_{1\alpha}$ to TXB_2 ratio tended to be higher following the HI-LO and IN-LO diets than following the LO-HI and

TABLE 5

Mean Plasma Phosphatidylcholine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
16:0	32.4 ± 3.0[a]	33.7 ± 2.7[a]	31.8 ± 2.7[a]	33.7 ± 3.0[a]
18:0	14.3 ± 0.8[a]	15.3 ± 0.8[b]	14.7 ± 0.8[a,b]	16.3 ± 0.8[c]
18:1	14.4 ± 1.1[a]	8.8 ± 0.8[b]	14.9 ± 0.8[a]	9.4 ± 1.1[b]
18:2n-6	22.3 ± 1.6[a]	26.6 ± 1.3[b]	20.9 ± 1.3[a]	25.7 ± 1.6[b]
18:3n-3	0.2 ± 0.1[a]	0.3 ± 0.1[b]	0.4 ± 0.1[b]	0.7 ± 0.1[c]
20:3n-6	2.3 ± 0.5[a]	1.4 ± 0.3[b]	1.9 ± 0.3[a]	1.4 ± 0.5[b]
20:4n-6	7.2 ± 1.0[a]	7.0 ± 0.8[a]	7.0 ± 0.8[a]	5.8 ± 1.0[b]
20:5n-3	0.3 ± 0.2[a]	0.3 ± 0.2[a]	0.8 ± 0.2[b]	0.6 ± 0.2[c]
22:4n-6	0.2 ± 0.1[a]	0.1 ± 0.1[b]	0.1 ± 0.1[b]	0.1 ± 0.1[b]
22:5n-3	0.4 ± 0.2[a]	0.4 ± 0.2[a]	0.6 ± 0.2[b]	0.5 ± 0.2[a,b]
22:6n-3	2.0 ± 0.5[a]	1.7 ± 0.5[a]	2.0 ± 0.5[a]	1.6 ± 0.5[a]

[a]Values in the same row with the same superscript letter (a-c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.

TABLE 6

Mean Plasma Phosphatidylethanolamine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
16:0	11.9 ± 1.9[a]	12.1 ± 1.6[a]	11.1 ± 1.6[a]	11.1 ± 1.9[a]
18:0	28.3 ± 2.2[a]	31.1 ± 1.9[b]	28.2 ± 1.9[a]	33.0 ± 2.2[b]
18:1	18.0 ± 1.9[a]	11.6 ± 1.9[b]	18.2 ± 1.9[a]	13.0 ± 1.9[b]
18:2n-6	11.9 ± 2.4[a]	15.8 ± 2.2[b]	11.5 ± 2.2[a]	14.9 ± 2.4[b]
18:3n-3	0.3 ± 0.2[a]	0.5 ± 0.2[b]	0.6 ± 0.2[b]	0.9 ± 0.2[c]
20:3n-6	1.2 ± 0.2[a]	0.9 ± 0.2[b]	1.2 ± 0.2[a]	0.9 ± 0.2[b]
20:4n-6	17.3 ± 2.7[a]	16.7 ± 2.4[a]	16.2 ± 2.4[a]	15.1 ± 2.7[a]
20:5n-3	0.4 ± 0.2[a]	0.3 ± 0.2[a]	0.9 ± 0.2[b]	0.8 ± 0.2[b]
22:4n-6	0.5 ± 0.2[a]	0.4 ± 0.2[a,b]	0.3 ± 0.2[b]	0.4 ± 0.2[a,b]
22:5n-3	0.9 ± 0.3[a]	1.0 ± 0.3[a,b]	1.1 ± 0.3[a,b]	1.3 ± 0.3[b]
22:6n-3	4.5 ± 1.3[a]	4.4 ± 1.3[a]	5.0 ± 1.3[a]	4.0 ± 1.3[a]

[a]Values in the same row with the same superscript letter (a-c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.

TABLE 7

Mean Plasma Alkenkylacyl Glycerophosphoethanolamine Fatty Acid Levels Following Different Experimental Diets[a]

Fatty acid	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
	mol% of total fatty acids			
18:1	8.9 ± 1.6[a]	5.5 ± 1.3[b]	8.1 ± 1.3[a]	5.0 ± 1.6[b]
18:2n-6	14.8 ± 2.9[a,b]	17.7 ± 2.6[b,c]	12.4 ± 2.6[a]	18.9 ± 2.9[c]
18:3n-3	0.3 ± 0.2[a]	0.5 ± 0.2[b]	0.7 ± 0.2[b,c]	0.9 ± 0.2[c]
20:3n-6	2.0 ± 0.3[a]	1.3 ± 0.3[c]	1.8 ± 0.3[a,b]	1.5 ± 0.3[b,c]
20:4n-6	45.1 ± 3.1[a]	44.2 ± 2.9[a]	44.3 ± 2.9[a]	42.7 ± 3.1[a]
20:5n-3	1.4 ± 0.5[a]	1.5 ± 0.5[a]	3.4 ± 0.5[b]	2.9 ± 0.5[b]
22:4n-6	2.5 ± 0.3[a]	2.8 ± 0.3[b]	2.2 ± 0.3[a]	2.4 ± 0.3[a]
22:5n-3	4.5 ± 0.8[a]	5.5 ± 0.8[b]	5.3 ± 0.8[b]	6.1 ± 0.8[b]
22:6n-3	12.7 ± 1.3[a]	13.9 ± 1.3[a]	13.6 ± 1.3[a]	12.9 ± 1.3[a]

[a]Values in the same row with the same superscript letter (a-c) do not differ significantly ($P > 0.05$). All values are mean ± SD; n = 8; see Table 1 for abbreviations.

TABLE 8

Bleeding Times and Bleeding-Time Prostanoid Production Following the Experimental Diets[a]

	Experimental diets			
	LO-HI	IN-IN	IN-LO	HI-LO
Bleeding time (min)	5.00 ± 0.26[a]	4.94 ± 0.23[a]	5.27 ± 0.23[a]	5.07 ± 0.26[a]
Bleeding-time production of:				
TXB_2 (μg/L)	5.01 ± 0.60[a]	5.15 ± 0.52[a]	3.81 ± 0.52[a]	4.11 ± 0.60[a]
6-Keto-$PGF_{1\alpha}$ (pg/min)	2.90 ± 0.51[a]	3.64 ± 0.44[a,b]	3.84 ± 0.44[a,b]	4.70 ± 0.51[b]
6-Keto-$PGF_{1\alpha}$/TBX_2 ratio	0.81 ± 0.17[a,b]	0.74 ± 0.12[a]	1.08 ± 0.16[a,b]	1.52 ± 0.31[b]

[a]Values in the same row with the same superscript letter (a-b) do not differ significantly ($P > 0.05$). All values are mean ± SEM; n = 8; see Table 1 for abbreviations.

IN-IN diets; the ratio following the HI-LO diet was significantly ($P < 0.05$) higher than the ratio following the IN-IN diet.

DISCUSSION

Levels of 18:1n-9, 18:2n-6 and 18:3n-3 in platelet and plasma PL following the consumption of the experimental diets reflected the difference in the levels of these fatty acids in the diets. Similar effects of dietary fat sources on the fatty acid composition of platelet and plasma PL have been reported (8,21,27-29). The magnitude of the differences in the fatty acid composition of the platelet and plasma PL, however, was small compared to the differences in dietary fatty acid composition, especially for 18:3n-3. The small amount of 18:3n-3 incorporated into

the PL could be due to its preferential oxidation for energy purposes. Leyton *et al.* (30) found that 18:1n-9 and 18:3n-3 were oxidized more rapidly than other long-chain fatty acids. Restricted incorporation of 18:3n-3 into PL or competition for the elongase and desaturase enzymes present in the liver and other tissues also could be responsible for the limited changes observed in platelet and plasma fatty acid composition (12, 31).

The IN-LO and HI-LO diets, which had relatively low 18:2/18:3 ratios, were associated with significantly higher levels of platelet and plasma long-chain n-3 PUFA, in particular the level of 20:5n-3, which was double that following the LO-HI and IN-IN diets. These results confirm previous studies (9,12,21,27-29) which indicated that humans can desaturate and elongate 18:3n-3 to higher homologs and that desaturation-elongation varies with the level of 18:3n-3 in the diet (12,21,27-29,31,32) and its ratio to 18:2n-6 (12,33,34). However, the *in vivo* conversion of dietary 18:3n-3 to 20:5n-3 is not as effective in raising n-3 levels in platelet and plasma PL as is direct supplementation with 20:5n-3 (8,9). The fact that the IN-LO diet, which had a lower dietary 18:2/18:3 ratio than the IN-IN diet, resulted in significantly higher levels of platelet and plasma 20:5n-3 suggests that the higher 20:5n-3 levels associated with the lower 18:2/18:3 ratio could be due to the effective competition of 18:3n-3 for the Δ6 desaturase enzyme (35). Results of the present study also suggest that the 18:2/18:3 ratio has to be less than 6.9 to have a significant effect on the level of long-chain n-3 PUFA.

Neither the lower level of 18:3n-3 in the diet nor its ratio to 18:2n-6 had any consistent effect on the longer chain n-6 homologs in platelet or plasma PL. Lasserre *et al.* (34) also found that dietary 18:3n-3 at a level of 1.5% of total energy (slightly less than the levels in the IN-IN and IN-LO diets in the present study) had no effect on serum levels of n-6 PUFA. By contrast, supplementing subjects with 30 mL linseed oil per day (approximately 16 g/d of 18:3n-3, which was similar to the amount present in the HI-LO diet) (31) or 60 mL/d (32) led to significant decreases in plasma 20:4n-6 levels.

The dietary fat source also had no effect on plasma 22:6n-3 levels in the present study. Sanders and Younger (8), Renaud *et al.* (27) and Lasserre *et al.* (34) also found that dietary 18:3n-3 had no effect on plasma 22:6n-3 levels. Mest *et al.* (31), on the other hand, reported an increase in 22:6n-3 level following linseed oil supplementation.

Individual PL species were analyzed in the present study so as to avoid the possibility that a large amount of one fraction might conceal changes in the fatty acid composition of other fractions. The apparent rates of incorporation of individual fatty acids differed among the three PL species reported here. For instance, appreciably higher levels of 20:4n-6, 22:4n-6, 22:5n-3 and 22:6n-3 were incorporated into the PPE than into the PC or PE fractions. However, the pattern of change was similar for the three PL species. Likewise, changes in the fatty acid composition of platelet and plasma PL responded in a similar way to differences in dietary fatty acid composition in spite of major differences in the fatty acid composition of the platelet and plasma PL, *e.g.*, higher levels of 20:4n-6 and lower levels of 18:2n-6 and 22:6n-3 in platelets.

Renaud *et al.* (27) and Budowski *et al.* (32) reported significant decreases in platelet aggregability and platelet coagulant activity following an increase in 18:3n-3 intake. Borchgrevink *et al.* (36), on the other hand, failed to find any effect of a linseed oil supplement on bleeding time. A study similar to the present (37), however, reported longer bleeding times and higher bleeding-time prostacyclin production following a canola oil diet, which provided 2.7% of total energy as 18:3n-3, than following a mixed fat diet (0.4% of energy as 18:3n-3). However, sunflower oil (0.3% of energy as 18:3n-3) produced a similar response to that of canola oil. In the present study, the experimental diets did not affect bleeding times even though bleeding-time production of 6-keto-$PGF_{1\alpha}$ was significantly higher following the HI-LO diet, in which 18:3n-3 provided 4.6% of total energy, than following the LO-HI diet, in which 18:3n-3 provided only 0.3% of total energy. There is no obvious explanation for the higher production of 6-keto-$PGF_{1\alpha}$ on the HI-LO diet. In fact, the n-6 fatty acid level in plasma PC was significantly lower following the HI-LO diet; however, plasma PL fatty acid levels may not reflect vascular tissue PL composition, the assumed source of PGI in the bleeding-time blood analyzed in the present study. Although there were no differences in bleeding-time TXB_2 production due to diet, TXB_2 production tended to be lower following the HI-LO and IN-LO diets, both of which resulted in higher 20:5n-3 levels in platelet and plasma PL. Morita *et al.* (38) reported a strong inhibition of *in vitro* TXA_2 synthesis by human platelets as a result of adding 20:5n-3 to the medium. It has been suggested (6,39) that higher tissue levels of 20:5n-3 may inhibit prostanoid formation from 20:4n-6 through an effect on cyclooxygenase activity or the availability of 20:4n-6. However, Mest *et al.* (31) found no relation between 20:5n-3 levels in serum PL and *in vitro* TXA_2 production by platelets. The ratio of 6-keto-$PGF_{1\alpha}$/TXB_2 was highest following the HI-LO diet and lowest following the IN-IN diet; the ratio following the IN-LO diet was intermediate between the HI-LO and the IN-IN regimens. Since TXA_2 promotes and PGI_2 inhibits platelet aggregation, the balance between *in vivo* production of TXA and PGI is important (39). It is interesting to note that the levels of 20:5n-3 in the PL fractions were significantly higher following the HI-LO and IN-LO diets than following the LO-HI and IN-IN diets but that the level of 20:4n-6 was significantly lower only for the platelet PE and the plasma PC fractions following the IN-LO and HI-LO diets, respectively.

A significant effect of diet on prostacyclin production and on the ratio of prostacyclin to thromboxane production under conditions where there was no significant change in the bleeding time requires comment. It is possible that the HI-LO diet may have an antithrombotic effect (on the basis of altered prostanoid production) without producing an increased tendency to bleed, a situation that would be very desirable from the point-of-view of coronary heart disease. On the other hand, it is possible that the apparent beneficial effect of the HI-LO diet, in terms of prostanoid production, could be balanced by changes in other factors, not measured in this study, so that the overall antithrombotic effect may not be as significant as the changes in prostanoid production would suggest. Which of these alternative interpretations of our results is more pertinent will still require further investigation.

The results of the present study indicate that dietary 18:3n-3 intake has an appreciable effect on the fatty acid

composition of platelet and plasma PL. The ratio of 18:2/18:3 in the diet appears especially important in influencing n-3 PUFA patterns. The changes in the production of TXB_2 and 6-keto-$PGF_{1\alpha}$ were found to coincide with the differences in the n-3 PUFA patterns of platelet and plasma PL.

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Effect of Diets Rich in Linoleic or α-Linolenic Acid on Phospholipid Fatty Acid Composition and Eicosanoid Production in Atlantic Salmon (*Salmo salar*)

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Atlantic salmon post-smolts were fed diets rich in linoleic acid (sunflower oil, SO), α-linolenic acid (linseed oil, LO) or long-chain polyunsaturated fatty acids (fish oil, FO) for a period of 12 wk. In the liver phospholipids of fish fed SO, the levels of 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 were significantly elevated compared to both other treatments. In choline phospholipids (CPL), ethanolamine phospholipids (EPL) and phosphatidylserine (PS) the levels of 22:4n-6 and 22:5n-6 were significantly elevated in fish fed SO. In liver phospholipids from fish fed LO, 18:2n-6, 20:2n-6 and 20:3n-6 were significantly elevated but 20:4n-6, 22:4n-6 and 22:5n-6 were similar or significantly decreased compared to fish fed FO. Liver phospholipids from fish fed LO had increased 18:3n-3 and 20:4n-3 compared to both other treatments while EPL and phosphatidylinositol (PI) also had increased 20:5n-3. In fish fed LO, 22:6n-3 was significantly reduced in CPL, PS and PI compared to fish fed FO. Broadly similar changes occurred in gill phospholipids. Production of 12-lipoxygenase metabolites in isolated gill cells stimulated with the Ca^{2+}-ionophore A23187 were significantly reduced in fish fed either SO or LO compared to those fed FO. However, the ratio 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE)/12-hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid (12-HEPE) was significantly elevated in stimulated gill cells from SO-fed fish. Although mean values of thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) were increased in fish fed SO, they were not significantly different from those of the other two treatments. *Lipids 28,* 819–826 (1993).

The so-called essential fatty acids, linoleic acid (18:2n-6) and linolenic acid (18:3n-3), are the precursors of the long-chain n-6 and n-3 species which are important components of the phospholipid bilayer of cell membranes. It is generally accepted that both the n-6 and n-3 fatty acids are metabolized by the same sequence of desaturating and elongating enzymes (1). In these pathways the desaturation steps are generally rate-limiting while elongation is rapid (2), and thus competition between substrate fatty acids at desaturase binding sites will determine the nature of the resulting polyunsaturated fatty acids (PUFA) and ultimately the composition of cellular membranes. In competitive terms, the n-3 PUFA are much more potent inhibitors of n-6 PUFA metabolism than *vice versa* although the relative concentrations of the two substrates will also determine the products that result (3,4). Many species of freshwater fish, including Atlantic salmon (*Salmo salar*), possess the enzymes necessary to elongate and desaturate 18:3n-3 to docosahexaenoic acid (DHA, 22:6n-3) (5) and are capable of metabolizing 18:2n-6 similarly, resulting in increased arachidonic acid (AA; 20:4n-6) in membrane phospholipids (6).

In mammals, AA is the major precursor for biologically active eicosanoids and in humans consuming a typical "Western-type" diet, overproduction of AA-derived eicosanoids may explain the prevalence of the many inflammatory conditions occurring in the developed world (7). Fish provide a useful model system for the study of eicosanoid metabolism since, although AA may be the preferred eicosanoid substrate (8,9), fish contain considerable amounts of eicosapentaenoic acid (EPA, 20:5n-3) and DHA, which can attenuate the production and efficacy of AA-derived eicosanoids (10). EPA can be metabolized by both cyclooxygenase and lipoxygenase enzymes resulting in products of lower bioactivity when compared to their AA homologues (11,12). EPA can also competitively inhibit the prostaglandin synthetase enzyme complex, thereby reducing production of AA-derived prostanoids (13). A number of recent studies have suggested that feeding oils rich in 18:3n-3 can reduce production of AA-derived eicosanoids by increasing levels of EPA in membrane phospholipids (14,15). In addition 18:3n-3 can directly inhibit cyclooxygenase activity (16). In previous studies with salmon we have shown that a high level of dietary 18:2n-6 can result in development of a severe cardiomyopathy involving active necrosis of both atrium and ventricle (17) and altered eicosanoid metabolism in blood leucocytes and gill cells (18).

The objective of the present study was to investigate the metabolism of 18:2n-6 and 18:3n-3 in salmon fed diets containing either sunflower oil (SO), linseed oil (LO) or fish oil (FO) by measuring phospholipid fatty acid compositions in liver and gill. The 12-lipoxygenase products and the cyclooxygenase products prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) were measured in isolated gill cells stimulated with the calcium ionophore A23187.

MATERIALS AND METHODS

Animals and diets. Three hundred and thirty Atlantic salmon S1 smolts (salmon undergoing transition to seawater in one year) were obtained from the S.O.A.F.D. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and distributed randomly into three tanks of 2000 L capacity each, which were supplied with seawater at a rate of 26 L/min. The fish (mean weight *ca.* 86 g) were subject to natural photoperiod, and the water temperature during the experimental period (August–November) varied from 15–10°C. Diets were supplied by automatic feeders which were adjusted to provide 20 g/kg biomass per day. Fish were weighed every 28 d and the ration adjusted accordingly.

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; CPL, choline phospholipids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPL, ethanolamine phospholipids; FO, fish oil; HDHE, hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid; HEPE, hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid; HETE, hydroxy-5, 8, 10, 14-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; LO, linseed oil; PGE_2, prostaglandin E_2; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SO, sunflower oil; TLC, thin-layer chromatography; TXB_2, thromboxane B_2; UV, ultraviolet.

The diets were formulated to meet the nutritional requirements of salmonid fish (19) and contained 47% protein and 16% lipid. The composition of the basal diet has been described in detail previously (20) and contained fishmeal (650 g/kg) (LT94, Ewos Ltd., Westfield, Lothian, Scotland), pre-cooked starch (150 g/kg), vitamin mix (10 g/kg), mineral mix (24 g/kg), α-cellulose (65.5 g/kg) and choline chloride (4 g/kg). The dietary lipid (100 g/kg) was supplied either as fish oil (Fosol, Seven Seas Ltd., Hull, United Kingdom), sunflower oil (Tesco, Cheshunt, United Kingdom) or linseed oil (ICN Biomedical Ltd., High Wycombe, United Kingdom). An antioxidant mix (0.4 g/kg) was mixed with the oil before adding to the other diet components (20). The fatty acid compositions of the diets are shown in Table 1.

Lipid extraction and fatty acid analysis. Samples were collected after 12 wk of the dietary trial and stored at −80°C until analyzed. Lipids were extracted from liver and gill tissue by the method of Folch *et al.* (21). Total lipid extracts were separated into choline phospholipids (CPL), ethanolamine phospholipids (EPL), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions by thin-layer chromatography (TLC) as described by Vitiello and Zanetta (22). The plates were sprayed with 0.1% 2′,7′-dichlorofluorescein in 97% methanol containing 0.05% butylated hydroxytoluene (BHT), and the lipid bands were visualized under ultraviolet (UV) light. Acid-catalyzed transmethylation was carried out overnight at 50°C as described by Christie (23). The fatty acid methyl esters were separated and quantified by gas–liquid chromatography (Carlo Erba Vega 6000, Fisons Ltd., Crawley, United Kingdom) on a 50 m × 0.32 mm capillary column (CP-Wax 51, Chrompak Ltd., London, United Kingdom). Hydrogen was used as carrier gas, and temperature programming was from 50°C at 35°C/min to 150°C and then to 225°C at 2.5°C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (24).

Preparation of isolated gill cells. The procedure used for isolating gill cells has been described in detail previously (18). Minced gill filaments were incubated in a Hanks' medium specially formulated for use with salmonid fish (25) containing 0.1% collagenase (type IV, Sigma Chemical Co., Poole, United Kingdom) for 45 min at room temperature with constant stirring. After filtering through nylon gauze, the cells were collected by centrifugation at 400 × *g* for 2 min, washed twice in Hanks' medium and finally resuspended in 1 mL of the same medium containing 1 mM $CaCl_2$.

Ionophore challenge and eicosanoid extraction. Isolated gill cells prepared as described above were placed in glass tubes pre-coated with Sigmacote and incubated in a shaking water bath at 18°C for 10 min. Calcium ionophore was added in 2 μL of dimethyl sulfoxide at a final concentration of 10 μM and the incubation continued for a further 20 min. The cells were sedimented by centrifugation

TABLE 1

Fatty Acid Composition of Diets

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
14:0	6.6	1.8	1.8
16:0	14.8	9.5	9.1
18:0	2.4	4.3	3.5
Total saturated[a]	24.6	15.9	14.9
16:1n-7	5.1	2.0	2.0
18:1n-9	9.7	17.8	17.4
18:1n-7	2.0	1.0	1.2
20:1n-9	10.5	3.1	3.1
22:1n-11	16.6	4.8	4.4
24:1	1.0	0.6	0.5
Total monoenoic[b]	45.2	29.5	28.7
18:2n-6	1.4	40.3	12.2
18:3n-6	0.2	0.1	t[d]
20:2n-6	0.3	0.1	0.1
20:3n-6	0.1	t	t
20:4n-6	0.6	0.5	0.5
Total n-6[c]	2.8	41.1	12.9
18:3n-3	1.3	0.5	31.8
18:4n-3	3.1	1.0	0.9
20:4n-3	0.7	0.2	0.2
20:5n-3	6.5	3.6	3.4
22:5n-3	0.9	0.5	0.5
22:6n-3	8.8	4.9	4.5
Total n-3	21.3	10.7	41.3
Total PUFA[e]	25.0	51.8	54.2
n-3/n-6 ratio	7.6	0.3	3.2

[a]Includes 15:0, 17:0, 20:0 and 22:0.
[b]Includes 20:1n-11, 20:1n-7 and 22:1n-9.
[c]Includes 22:4n-6 and 22:5n-6.
[d]t = Trace value <0.05%.
[e]PUFA, polyunsaturated fatty acids.

(12000 × *g*, 2 min) and the eicosanoids extracted from the supernatant using C_{18} Sep-Pak minicolumns (Millipore Ltd., Watford, United Kingdom) according to Powell (26)

High-performance liquid chromatography (HPLC). The hydroxy acids 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE), 12-hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid (12-HEPE) and 14-hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid (14-HDHE) were separated and quantified by reverse-phase HPLC using a Spherisorb 5 μm octadecyl silane (ODS 2) column (25 cm × 4.6 mm, Anachem, Luton, United Kingdom). The chromatographic system was equipped with Waters Model M-45 pumps (Waters Chromatography, Warford, United Kingdom) and the effluent was monitored at 235 nm using a Pye-Unicam LC-UV detector (Pye-Unicam, Cambridge, United Kingdom). An isocratic solvent system containing acetonitrile/methanol/water/acetic acid (40:29:30:0.5, by vol) was used at a flow rate of 1 mL/min. Quantification was based on use of external standards of 12-HETE and 12-HEPE. Identification of 14-HDHE was done as described previously (18).

Measurement of PGE_2 and TXB_2. PGE_2 and TXB_2, the stable metabolite of thromboxane A_2, were measured by enzyme immunoassay using kits supplied by Cascade Biochemicals Ltd. (Reading, United Kingdom).

Materials. All solvents were of HPLC grade and were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, United Kingdom). TLC plates (20 cm × 20 cm × 0.25 mm), pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany). 12(*R,S*)-HETE and 12(*S*)-HEPE were obtained from Cascade Biochemicals Ltd. (Reading, United Kingdom). Sigmacote and A23187 were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom).

Statistical analysis. Significance of difference ($P < 0.05$) between dietary treatments was determined by analysis of variance using a Statgraphics (system 3.0) computer package. Data which were identified as nonhomogeneous were subjected to either arcsine square root or log transformation before analysis. Differences between means were determined by Tukey's test.

RESULTS

The fatty acid compositions of liver CPL are shown in Table 2. Total monoenoic fatty acids were significantly reduced in SO-fed fish compared to FO-fed fish. In SO-fed fish 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 were all significantly increased compared to both other dietary treatments. Fish fed LO had significantly increased 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA but significantly reduced 22:5n-6 compared to fish fed FO. LO fish had significantly increased 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3 compared to both other dietary treatments but had significantly reduced DHA compared to FO-fed fish. In fish fed SO 18:3n-3, 20:4n-3, EPA, 22:5n-3 and total n-3 PUFA were significantly reduced compared with both other dietary treatments while DHA was significantly reduced compared to FO-fed fish. The n-3/n-6 PUFA ratio was significantly different in the three dietary treatments with the highest value in FO-fed fish and the lowest in SO-fed fish.

The fatty acid compositions of liver EPL are shown in Table 3. Total monoenoic fatty acids were significantly different for each dietary treatment with the highest levels in FO-fed fish and the lowest levels in SO-fed fish. Also, 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA were

TABLE 2

Fatty Acid Compositions of Choline Phospholipids from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	29.3 ± 1.6	29.9 ± 3.5	31.7 ± 3.7
Total monoenoic	16.0 ± 1.6^{b}	13.2 ± 0.8^{c}	14.6 ± 1.1^{b,c}
18:2n-6	1.1 ± 0.3^{d}	10.6 ± 1.7^{b}	4.0 ± 0.5^{c}
18:3n-6	t^{d}	0.5 ± 0.3^{b}	0.2 ± 0.0^{c}
20:2n-6	0.3 ± 0.1^{d}	1.7 ± 0.5^{b}	0.6 ± 0.1^{c}
20:3n-6	0.3 ± 0.1^{d}	6.4 ± 1.0^{b}	2.0 ± 0.5^{c}
20:4n-6	0.7 ± 0.1^{c}	5.5 ± 2.2^{b}	0.6 ± 0.1^{c}
22:4n-6	0.1 ± 0.0^{c}	0.7 ± 0.4^{b}	0.1 ± 0.0^{c}
22:5n-6	0.3 ± 0.1^{c}	0.7 ± 0.3^{b}	0.1 ± 0.0^{c}
Total n-6	2.8 ± 0.3^{d}	26.0 ± 0.4^{b}	7.4 ± 0.4^{c}
18:3n-3	0.3 ± 0.1^{c}	0.1 ± 0.0^{d}	3.6 ± 1.2^{b}
18:4n-3	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.7 ± 0.2^{b}
20:3n-3	t^{c}	t^{c}	0.4 ± 0.1^{b}
20:4n-3	0.8 ± 0.2^{c}	0.2 ± 0.1^{d}	2.9 ± 0.8^{b}
20:5n-3	9.8 ± 1.0^{b}	3.8 ± 0.4^{c}	9.5 ± 1.2^{b}
22:5n-3	3.0 ± 0.6^{b}	2.0 ± 0.5^{c}	3.3 ± 0.4^{b}
22:6n-3	35.9 ± 2.6^{b}	23.8 ± 3.2^{c}	25.5 ± 1.7^{c}
Total n-3	49.6 ± 1.8^{b}	29.7 ± 3.1^{c}	45.8 ± 2.2^{b}
Total PUFA	52.4 ± 1.8	55.7 ± 3.2	53.1 ± 2.1
n-3/n-6	18.0 ± 1.6^{b}	1.2 ± 0.1^{d}	6.2 ± 0.5^{c}
20:4/20:5	0.1 ± 0.0^{c}	1.5 ± 0.6^{b}	0.1 ± 0.0^{c}

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

TABLE 3

Fatty Acid Compositions of Ethanolamine Phospholipids from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	12.6 ± 0.7	12.7 ± 0.6	13.6 ± 1.4
Total monoenoic	25.2 ± 1.8[b]	15.5 ± 2.5[d]	21.1 ± 0.4[c]
18:2n-6	1.7 ± 0.4[d]	12.0 ± 2.1[b]	4.7 ± 1.1[c]
18:3n-6	0.1 ± 0.0	0.2 ± 0.1	t
20:2n-6	0.4 ± 0.1[d]	2.3 ± 0.5[b]	0.7 ± 0.1[c]
20:3n-6	0.3 ± 0.1[d]	5.1 ± 0.6[b]	1.5 ± 0.2[c]
20:4n-6	1.3 ± 0.4[c]	9.6 ± 2.7[b]	1.1 ± 0.1[c]
22:4n-6	t[c]	0.8 ± 0.4[b]	t[c]
22:5n-6	0.5 ± 0.0[c]	1.0 ± 0.3[b]	0.3 ± 0.1[d]
Total n-6	4.2 ± 0.4[d]	30.9 ± 2.5[b]	8.1 ± 1.1[c]
18:3n-3	0.8 ± 0.2[c]	0.3 ± 0.1[d]	3.0 ± 0.6[b]
20:3n-3	t[c]	t[c]	0.7 ± 0.1[b]
20:4n-3	0.5 ± 0.1[c]	t[d]	1.7 ± 0.4[b]
20:5n-3	8.8 ± 1.2[c]	3.6 ± 1.0	11.1 ± 1.1[b]
22:5n-3	2.1 ± 0.4[b,c]	1.8 ± 0.4[c]	2.8 ± 0.4[b]
22:6n-3	41.9 ± 1.9[b]	32.5 ± 2.7[c]	36.0 ± 0.9[c]
Total n-3	54.1 ± 0.8[b]	38.2 ± 2.2[c]	55.4 ± 0.3[b]
Total PUFA	58.3 ± 1.2[d]	69.2 ± 2.7[b]	63.5 ± 1.4[c]
n-3/n-6	12.9 ± 1.2[b]	1.3 ± 0.1[d]	6.9 ± 0.9[c]
20:4/20:5	0.2 ± 0.1[c]	2.9 ± 1.4[b]	0.1 ± 0.0[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

significantly affected by dietary treatment with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. Levels of 22:5n-6 were also significantly different in each dietary group with highest values in SO-fed fish and lowest in LO-fed fish. AA and 22:4n-6 were significantly increased in fish fed SO compared to both other dietary treatments. Also, 18:3n-3, 20:4n-3 and EPA were significantly different in each dietary treatment with highest levels in LO-fed fish and lowest in SO-fed fish. 22:5n-3 was significantly increased in LO-fed fish compared to SO-fed fish whereas DHA was significantly greater in FO-fed fish compared to both other treatments. Total n-3 PUFA were significantly lower in SO-fed fish while total PUFA were significantly different in each dietary treatment with the highest levels in SO-fed fish and the lowest in FO-fed fish. The ratio of n-3/n-6 PUFA was significantly different in each dietary treatment with the highest value in FO-fed fish and the lowest in SO-fed fish. The AA/EPA ratio of eicosanoid precursors was significantly increased in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PS are shown in Table 4. Total saturated fatty acids were significantly increased whereas total monoenoic fatty acids were significantly decreased in fish fed SO and LO compared to those fed FO. The level of 20:3n-6 was significantly different in each dietary treatment with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA and 22:5n-6 were also significantly different in each treatment with the highest levels in SO-fed fish and the lowest in LO-fed fish. 18:2n-6, 20:2n-6, 22:4n-6 and total n-6 PUFA were all significantly increased in fish fed SO compared to the other dietary treatments. Levels of 18:3n-3 and 20:4n-3 were significantly greater in fish fed LO than in the two other treatments while EPA was significantly reduced in SO-fed fish compared to LO-fed fish. Total n-3 PUFA were significantly reduced in SO-fed fish compared to both other treatments while DHA was reduced in fish fed SO compared to those fed FO. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PI are shown in Table 5. Total saturated fatty acids were significantly elevated in fish fed SO and LO compared with those fed FO. Total monoenoic fatty acids were significantly different in all treatments with the highest levels in fish fed FO and the lowest in those fed SO. Levels of 18:2n-6 and total n-6 PUFA were significantly increased in SO-fed fish compared to the other two treatments whereas 20:3n-6 was significantly greater in LO-fed fish compared to the other treatments. AA was significantly different in all dietary treatments with the highest level in SO-fed fish and the lowest level in LO-fed fish. Also, 18:3n-3, 20:4n-3 and total n-3 PUFA were significantly reduced in fish fed SO compared to the other two treatments. EPA was significantly different in all treatments with the highest level in LO-fed fish and the lowest in SO-fed fish. Levels of 22:5n-3 and DHA were significantly different in all treatments with the highest levels in FO-fed fish and the lowest in SO-fed fish. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in fish fed SO compared to both other treatments.

The major PUFA in gill CPL and EPL are shown in Figure 1. in CPL 18:2n-6 and 20:3n-6 were significantly

TABLE 4

Fatty Acid Compositions of Phosphatidylserine from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	31.7 ± 1.5[c]	35.5 ± 1.4[b]	35.8 ± 1.8[b]
Total monoenes	10.3 ± 0.8[b]	6.2 ± 0.5[c]	6.7 ± 1.0[c]
18:2n-6	0.5 ± 0.1[c]	1.5 ± 0.2[b]	0.6 ± 0.1[c]
18:3n-6	0.2 ± 0.2	0.2 ± 0.1	t
20:2n-6	0.2 ± 0.0[c]	1.0 ± 0.3[b]	0.3 ± 0.1[c]
20:3n-6	0.2 ± 0.1[d]	1.6 ± 0.5[b]	0.6 ± 0.1[c]
20:4n-6	0.5 ± 0.1[c]	1.7 ± 0.3[b]	0.2 ± 0.1[d]
22:4n-6	t[c]	0.8 ± 0.5[b]	t[c]
22:5n-6	0.6 ± 0.1[c]	1.1 ± 0.4[b]	0.3 ± 0.0[d]
Total n-6	2.0 ± 0.2[c]	8.0 ± 0.7[b]	2.0 ± 0.1[c]
18:3n-3	0.4 ± 0.1[b,c]	0.2 ± 0.1[c]	0.6 ± 0.2[b]
18:4n-3	0.4 ± 0.1[b]	t[c]	t[c]
20:4n-3	t[c]	t[c]	0.5 ± 0.1[b]
20:5n-3	1.4 ± 0.5[b,c]	0.7 ± 0.3[c]	1.4 ± 0.3[b]
22:5n-3	2.7 ± 0.4	3.2 ± 0.8	3.8 ± 0.6
22:6n-3	47.3 ± 1.6[b]	43.2 ± 2.3[c]	44.6 ± 0.7[b,c]
Total n-3	52.3 ± 0.3[b]	47.2 ± 1.7[c]	50.9 ± 0.7[b]
Total PUFA	54.3 ± 1.0[b,c]	55.2 ± 1.3[b]	52.9 ± 0.7[c]
n-3/n-6	25.9 ± 2.1[b]	5.9 ± 0.6[c]	25.8 ± 1.3[c]
20:4/20:5	0.4 ± 0.2[c]	3.1 ± 1.6[b]	0.2 ± 0.1[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

different in all dietary treatments with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA was significantly increased and EPA significantly decreased in SO-fed fish compared to the other treatments. Consequently, the AA/EPA ratio was significantly greater in SO fish compared to the other two treatments. DHA and total n-3 PUFA were significantly decreased in SO-fed fish compared to both other treatments. Largely similar effects occurred in gill EPL except that 20:3n-6 was significantly greater in SO-fed fish compared to both other treatments and DHA was significantly reduced in SO-fed fish compared to FO-fed fish.

The major PUFA of gill PS and PI are shown in Figure 2. In gill PS 18:2n-6 and 20:3n-6 were significantly

TABLE 5

Fatty Acid Compositions of Phosphatidylinositol from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	33.3 ± 1.6[c]	38.1 ± 0.5[b]	38.4 ± 1.1[b]
Total monoenoic	12.5 ± 1.4[b]	6.7 ± 0.7[d]	9.2 ± 0.6[c]
18:2n-6	0.5 ± 0.1[c]	1.4 ± 0.3[b]	0.6 ± 0.1[b]
20:2n-6	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
20:3n-6	2.0 ± 0.3[c]	1.8 ± 0.7[c]	5.8 ± 1.2[b]
20:4n-6	28.4 ± 0.3[c]	41.8 ± 0.4[b]	21.0 ± 1.2[d]
Total n-6	31.5 ± 0.2[c]	46.1 ± 0.5[b]	28.3 ± 1.7[c]
18:3n-3	0.4 ± 0.1[b]	t[c]	0.5 ± 0.1[b]
20:4n-3	0.3 ± 0.1[b]	t[c]	0.5 ± 0.1[b]
20:5n-3	4.9 ± 0.9[c]	0.7 ± 0.3[d]	10.1 ± 2.5[b]
22:5n-3	2.2 ± 0.3[b]	0.6 ± 0.1[d]	1.7 ± 0.2[c]
22:6n-3	12.4 ± 1.2[b]	4.7 ± 0.5[d]	8.1 ± 0.4[c]
Total n-3	19.7 ± 0.4[b]	6.2 ± 0.6[c]	21.5 ± 2.9[b]
Total PUFA	51.4 ± 0.7[b,c]	52.2 ± 1.1[b]	49.7 ± 1.1[c]
n-3/n-6	0.6 ± 0.0[b]	0.1 ± 0.0[c]	0.7 ± 0.2[b]
20:4/20:5	7.0 ± 2.2[c]	76.1 ± 28.2[b]	3.2 ± 2.2[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

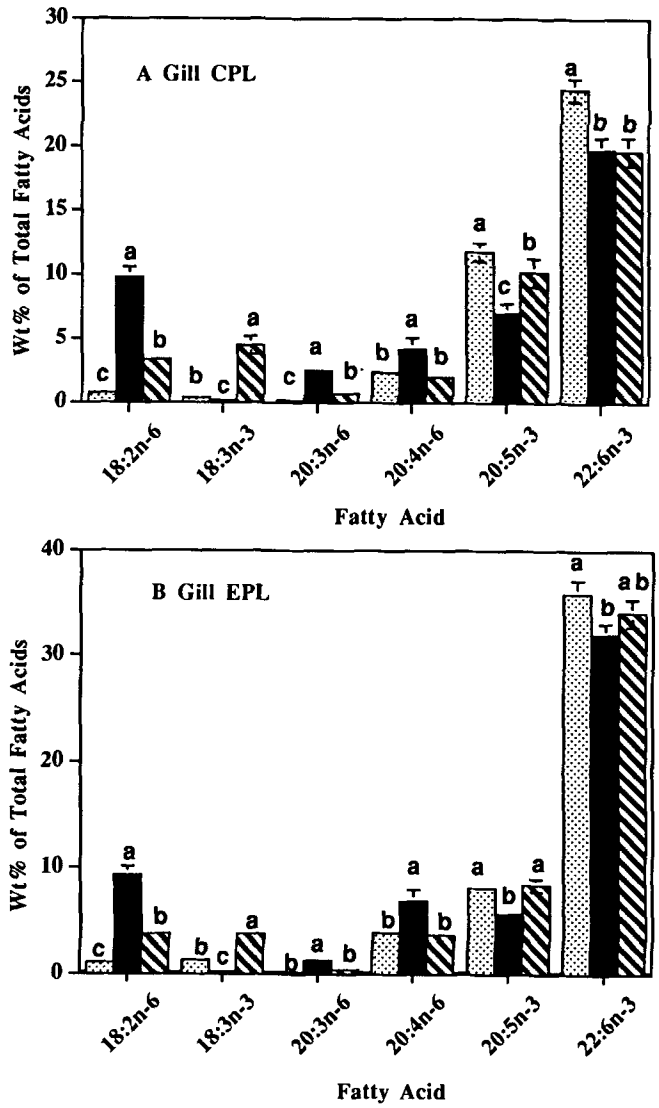

FIG. 1. The levels of the major polyunsaturated fatty acids of gill choline phospholipids (CPL) (A) and ethanolamine phospholipids (EPL) (B) from salmon fed diets containing either fish oil (dotted bars), sunflower oil (filled bars) or linseed oil (hatched bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

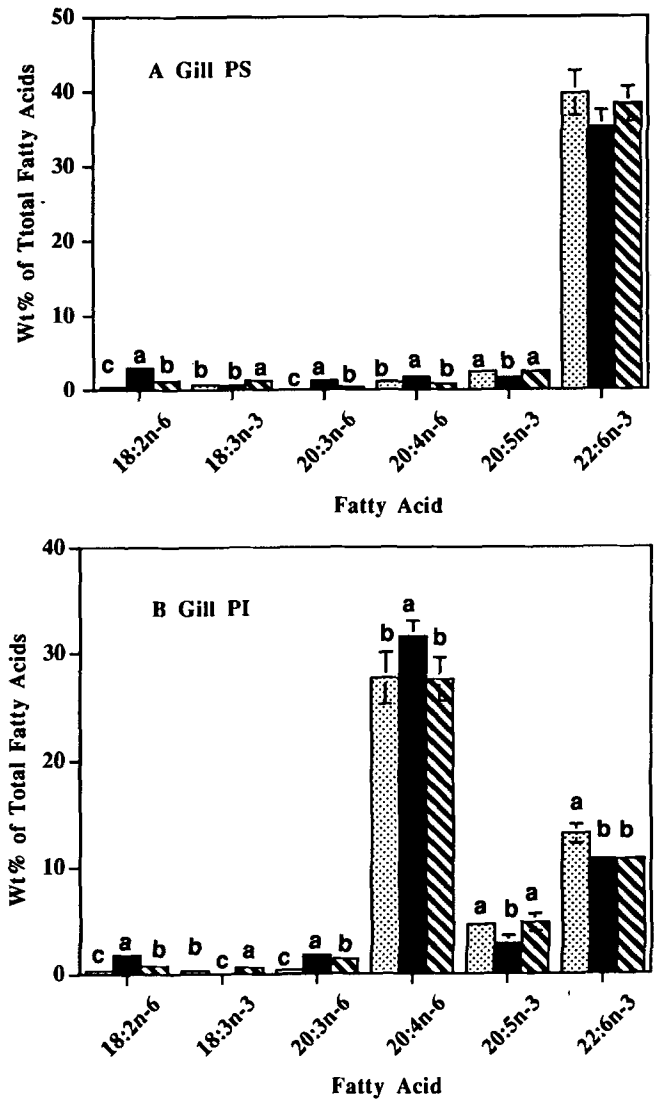

FIG. 2. Levels of the major polyunsaturated fatty acids of gill phosphatidylserine (PS) (A) and phosphatidylinositol (PI) (B) from salmon fed diets containing either fish oil (dotted bars), sunflower oil (filled bars) or linseed oil (hatched bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

different in all dietary treatments with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. AA was significantly increased and EPA significantly reduced in SO-fed fish, resulting in an increased AA/EPA ratio when compared to fish fed either FO or LO. In gill PI the results were largely similar to PS except that DHA was significantly greater in fish fed FO than in both other dietary treatments.

The production of eicosanoids by isolated gill cells stimulated by A23187 is shown in Table 6. The production of 12-HETE, 12-HEPE and 14-HDHE were all significantly reduced in fish fed both LO and SO compared to those fed FO. However, the ratio of 12-HETE/12-HEPE was significantly increased in fish fed SO compared to both other treatments. While mean values of both TXB_2 and PGE_2 were greatest in fish fed SO, they were not significantly different from either of the other dietary treatments.

DISCUSSION

Feeding SO results in increased levels of 18:2n-6, 20:2n-6, 20:3n-6 and AA in all liver phospholipids while 22:4n-6 and 22:5n-6 were increased in CPL, EPL and PS compared to both other treatments. 20:3n-6, the product of Δ6 desaturation and elongation of 18:2n-6, was also increased in fish fed LO, whereas AA, the product of Δ5 desaturation, was generally decreased in those fish compared to fish fed FO. Similarly 22:5n-6, the product of Δ4 desaturation and elongation of AA was decreased in fish fed LO compared to those fed FO. Therefore it appears that feeding LO, which contains 18:2n-6 and 18:3n-3 in a ratio

TABLE 6

Eicosanoids from Isolated Salmon Gill Cells Stimulated with the Ca^{2+}-Ionophore A23187

Eicosanoid[a]	Fish oil diet	Sunflower oil diet	Linseed oil diet
12-HETE	17.7 ± 9.6[b]	5.7 ± 2.9[c]	5.8 ± 0.5[c]
12-HEPE	45.2 ± 12.4[b]	7.8 ± 3.2[c]	21.2 ± 8.2[c]
12-HETE/12-HEPE	0.354 ± 0.094[c]	0.791 ± 0.189[b]	0.327 ± 0.138[c]
14-HDHE	34.1 ± 11.8[b]	6.8 ± 3.8[c]	10.4 ± 1.9[c]
TXB_2	142.8 ± 80.4	229.0 ± 120.9	172.0 ± 59.6
PGE_2	192.3 ± 54.0	807.3 ± 562.3	510.3 ± 177.9

[a]Values are mean ± SD from four fish per treatment. Values for 12-HETE, 12-HEPE and 14-HDHE are ng/mg protein, whereas those for TXB_2 and PGE_2 are pg/mg protein. [b,c]Values in the same row with different superscript letters are significantly different ($P < 0.05$). HETE, hydroxy-5, 8, 10, 14-eicosatetraenoic acid; l HEPE, hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid; HDHE, hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid; TXB_2, thromboxane B_2; PGE_2, prostaglandin E_2.

of 1:3, has an inhibitory effect on Δ5 (and possibly Δ4) desaturase which is responsible for AA production. The ability of 18:3n-3 to reduce the conversion of 18:2n-6 to AA, by inhibition of Δ6 desaturase, has been recorded previously in mammals (27,28). The differences in 20:3n-6 and AA production by fish fed either SO or LO can be explained by considering the competitive influences of the different dietary fatty acid compositions. In SO-fed fish the high 18:2n-6 level makes AA production inevitable whereas the presence of 18:3n-3 in LO-fed fish results in competition which reduces 20:3n-6 production from 18:2n-6. At the same time Δ6 desaturation and elongation of 18:3n-3 results in increased 20:4n-3 in LO-fed fish, and this, coupled with reduced 20:3n-6, results in reduced AA production and an apparent inhibition of Δ5 desaturase. Similar competitive effects presumably operate to increase or reduce 22:5n-6 levels in SO- and LO-fed fish, respectively.

In a previous study using the related salmonid, the rainbow trout, no increase in elongated and desaturated products of 18:3n-3 were observed in the polar lipid fraction of fish fed 18:3n-3 enriched diets (29). However, in the present study with Atlantic salmon, although dietary EPA levels were similar in both SO and LO diets, the fatty acid compositions of phospholipid classes of both liver and gill showed decreased EPA in SO-fed fish, but LO-fed fish had EPA values greater than or equal to those of FO-fed fish. These results suggest that salmon are capable of EPA production from 18:3n-3 utilizing the pathways of Δ6 and Δ5 desaturation and elongation. Although some phospholipid classes from liver of LO-fed fish had elevated 22:5n-3, there was no apparent increase in DHA compared to fish fed FO. Previous studies with Atlantic salmon parr have demonstrated that they are capable of converting 18:3n-3 to DHA (5) but in the larger post-smolts used in the present study Δ4 desaturation is either very low or, more likely, the requirement for DHA is met by dietary input.

Both AA and EPA can be metabolized by cyclooxygenase to yield prostaglandins of the 2 and 3 series and by lipoxygenase to yield leukotrienes of the 4 and 5 series and a number of HETE and HEPE isomers (14,30). It is generally regarded that EPA is a poorer substrate for cyclooxygenase than AA and thus acts as a competitive inhibitor (16). The biological activity of the EPA-derived prostanoids and leukotrienes is considerably less than that of their AA-derived equivalents (12,31) which explains the ability of so-called 'MaxEPA' preparations to attenuate many of the pathophysiological conditions which occur in humans (7). In the present study feeding LO decreases AA and increases EPA in tissue phospholipids of Atlantic salmon and might therefore be expected to alter the spectrum of eicosanoids produced by these fish.

Gill cells were chosen since they are known to possess a highly active 12-lipoxygenase (18,32) and also to contain cyclooxygenase activity (5). While overall production of 12-lipoxygenase products was greatest in fish fed FO, the ratio of 12-HETE/12-HEPE was increased in SO-fed fish and was similar in both FO- and LO-fed fish. Although no specific physiological role has been identified for products of 12-lipoxygenase, a number of recent studies have implicated 12-HETE as a modulator of ion channels (33,34). Clearly this activity would be particularly important in gills which, along with posterior kidney, are vital in controlling osmoregulation in fish. Diet-induced changes in gill lipoxygenase products might therefore affect the ability of salmon to adapt to varying salinity.

In a previous study Atlantic salmon given increasing dietary linoleic acid produced decreasing amounts of gill 12-lipoxygenase products (18). In the present study salmon fed LO also produced significantly less 12-lipoxygenase products compared to fish fed FO. A similar decrease was also observed in platelet 12-lipoxygenase products from rats fed 18:3n-3 compared to those fed FO (14). One possible explanation is that changes in membrane phospholipid fatty acid composition might affect the activity of phospholipase A_2 which provides the precursors for eicosanoid production. Recent studies have established that phospholipase A_2 activity can be either increased or decreased in different tissues as a result of decreasing the membrane n-3/n-6 PUFA ratio (35,36).

The tendency for the PI fraction to accumulate 20-carbon fatty acids in fish has resulted in the hypothesis that this phospholipid might be the source of precursor fatty acids for eicosanoid production (5,37). However, the ratio of 12-HETE/12-HEPE produced by stimulated gill cells in the current experiment was most similar to the AA/EPA ratio present in gill CPL (0.20, 0.61 and 0.20 for FO, SO and LO diets, respectively). Comparison of the precursor fatty acid ratios of both CPL and PI would make the former class the more likely source of lipoxygenase substrate fatty acids. A similar result has been recorded in mammalian platelets (38) and cultured umbilical cells (39).

The present study demonstrated that while diets rich in 18:2n-6 result in increased AA in membrane lipids of

salmon, the fatty acid compositions can be 'normalized' to the position in fish fed FO, by feeding 18:3n-3. In this context linseed oil could be useful, when used in conjunction with marine oils, as an inhibitor of inflammatory activity in fish.

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Dietary Fish Oil Inhibits Human Breast Carcinoma Growth: A Function of Increased Lipid Peroxidation

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Female athymic nude mice were implanted subcutaneously with human breast carcinoma MDA-MB231. Seven to ten days later, the mice were divided into groups and fed a purified diet containing the following types of fat (% of diet): (i) 20% corn oil (CO); (ii) 15% CO:5% fish (menhaden) oil (FO); (iii) 10% CO:10% FO; (iv) 5% CO:15% FO; (v) 1% CO:19% FO; and (vi) 1% CO:19% FO plus antioxidants (α-tocopherol acetate, 2000 IU/kg diet and tertiary butylhydroquinone, 2% of total fat). The linoleic acid levels (% of diet) of the groups were 12.0, 9.1, 6.2, 3.3, 0.9 and 0.9%, respectively. After 6–8 wk, the carcinomas were assessed for tumor volume (cm^3) and assayed for thiobarbituric acid reactive substances (TBARS). Human breast carcinoma growth was suppressed in mice consuming FO diets without antioxidants as compared to mice fed CO; the greater the amount of dietary FO fed, the greater the carcinoma growth suppression ($P < 0.05$). The addition of antioxidants to the FO diet significantly ($P < 0.05$) reversed the FO-induced carcinoma growth suppression. Concentrations of TBARS in the human breast carcinomas were increased in all the FO (without antioxidants) fed mice, compared to mice fed CO; the level of increase in TBARS was directly related to the increase in the level of FO fed ($P < 0.05$). The addition of antioxidants to the FO diet significantly ($P < 0.05$) reduced the concentration of TBARS in the breast carcinomas. Thus, these results provide evidence that dietary FO can significantly suppress growth of human breast carcinoma MDA-MB231, even in the presence of substantial amounts of linoleic acid (3.3–9.1%). The inhibitory effect of FO on growth of these carcinomas was associated with an increased concentration of TBARS in the tumor tissue. In conclusion, dietary FO induced suppression of human breast carcinoma growth is a function, at least in part, of an accumulation of lipid peroxidation products in the tumor tissues.
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Fish oil (FO), which contains elevated levels of long-chain ω3 polyunsaturated fatty acids, particularly eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), has been shown to suppress mammary gland tumorigenesis in rodent models (1–4). In addition, FO has been shown to inhibit the growth of human breast carcinomas maintained in immune-deficient animals (5–9). To explain how FO inhibits mammary tumorigenic processes, many investigators (2–4) have focused on EPA- and/or DHA-induced alterations in eicosanoid metabolism. Other investigators (10,11) suggested that dietary FO inhibits mammary tumorigenesis because it may not provide a sufficient amount of essential fatty acids, such as linoleic acid (18:2). Indeed, a direct relationship between the amount of dietary linoleic acid and mammary tumor growth has been clearly shown (12). In contrast, we have explored yet another potential mechanism, *i.e.*, that high levels of dietary FO inhibit mammary tumorigenesis by causing an increased accumulation of cytostatic and/or cytotoxic lipid peroxidation products in the tumor tissue (9).

In this communication, we further examine the relationship between dietary FO and growth of human breast carcinomas in athymic nude mice by pursuing the following objectives: (i) to determine whether or not high levels of dietary FO, in the presence of substantial amounts of linoleic acid, can inhibit growth of these tumors and (ii) whether or not there is a correlation between tumor growth and the quantity of lipid peroxidation products in the tumor tissues of mice fed FO.

MATERIALS AND METHODS

Animals and diet. One hundred and sixty-four female athymic nude mice (Harlan Sprague Dawley Inc., Madison, WI), 4–5-wk-old, were used in these studies. The mice were housed under aseptic conditions (enclosed overhead laminar flow hood, sterilized cages, bedding and water) in a temperature (24°C) and light-controlled (14 h/d) room. All experimental procedures pertaining to the athymic mice were performed under aseptic conditions. Autoclaved laboratory mouse chow (Purina Mills Inc., St. Louis, MO) was fed *ad libitum* before and up to 7–10 d after human breast carcinoma transplantation. Thereafter, mice were fed purified diets (Table 1) *ad libitum.* The percentages of the predominant fatty acids (1% or greater, manufacturer's specifications) of the dietary oils are shown in Table 2. The diets were prepared weekly in our laboratory, individually packed in small, plastic zipper-sealed bags of sufficient size for one day's feed, flushed with nitrogen, sealed and stored at −20°C. Mice were fed daily and nonconsumed food was discarded daily. Because the purified diets were not sterilized, antibiotics (bacityracin combined with streptomycin or neomycin, 1 g/L) were added to the distilled drinking water.

Human breast carcinoma transplantation. Palpable MDA-MB231 human breast carcinomas (American Type Culture Collection, Rockville, MD) were surgically excised from female athymic mice, cut into slices (2 × 4 mm, 0.1–0.3 mm thick) and implanted into recipient mice. Mice were anesthetized with sodium pentobarbital (60 μg/g, *i.p.*) prior to transplantation. An incision was made in the integument, the tumor slices were placed subcutaneously in the dorsum at distances from each other of at least 2 cm, 3–4 slices/mouse. Between one and three palpable human breast carcinomas were pooled to provide the tissue slices. Mice were placed on the experimental diets 7–10 d after carcinoma transplantation. This period allows the carcinoma grafts to become established in the host animals before the onset of experimental dietary treatments. Athymic mice bearing human breast carcinoma grafts

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Abbreviations: CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroquinone.

TABLE 1

Diet Composition

Ingredients[a]	Percent[b]
Fat[c]	20.00
Casein[d]	20.17
dl-Methionine	0.35
Dextrose	32.18
Sucrose	16.09
AIN mineral mix[e]	4.13
AIN vitamin mix[f]	1.18
Cellulose[g]	5.90

[a]All ingredients (dry components) were obtained from U.S. Biochemical Co. (Cleveland, OH) except sucrose (ICN Biochemicals, Inc., Costa Mesa, CA).
[b]High-fat diets (20% w/w) consisted of 20% corn oil or a fish oil (menhaden)/corn oil combination totaling 20%.
[c]Corn oil (tocopherol stripped) was obtained from U.S. Biochemical Co. Fish oil (menhaden) was obtained from Zapata Haynie Corp. (Reedville, VA).
[d]Vitamin-free, high-nitrogen (14.5%).
[e]The mineral mixture supplied, per kg diet: $CaHPO_4$, 20.65 g; NaCl, 3.06 g; potassium citrate, 9.09 g; K_2SO_4, 2.15 g; MgO, 991.20 mg; manganous carbonate, 144.55 mg; ferric citrate, 247.8 mg; zinc carbonate, 66.08 mg; cupric carbonate, 12.39 mg; KIO_3, 0.41 mg; sodium selenite, 0.41 mg; chromium potassium sulfate, 22.72 mg; sucrose, 4.87 g.
[f]The vitamin mixture provided, per kg diet: thiamine HCl, 7.08 mg; riboflavin, 708 mg; pyridoxine HCl, 8.26 mg; nicotinic acid, 35.40 mg; D-calcium pantothenate, 18.88 mg; folic acid, 2.36 mg; DL-α-tocopheryl acetate, 236.00 mg; cholecalciferol, 0.03 mg; menaquionine, 0.06 mg; sucrose, 11.48 g.
[g]Celufil, nonnutritive bulk.

TABLE 2

Predominant Fatty Acids (%)

Fatty acids[a]	Corn oil	Fish (menhaden) oil
Myristic (14:0)	—	6.7
Palmitic (16:0)	10.8	15.7
Palmitoleic (16:1)	—	8.7
Stearic (18:0)	2.1	2.7
Oleic (18:1)	26.5	14.3
Linoleic (18:2)	60.0	1.8
Linolenic (18:3)	—	1.5
Arachidonic (20:4)	—	2.2
Eicosapentaenoic (20:5)	—	15.5
Docosahexaenoic (22:6)	—	12.1

[a]Fatty acid concentrations <1% are not included.

were fed the experimental diets for a period of 6–8 wk. At the termination of the studies, all tumors were excised and their volume determined ($V = 4/3\ \pi\ r^3$) with a vernier caliper. Four separate studies were carried out; each study was a complete individual experiment.

Study 1. The experimental design of this study is provided in Table 3. The purpose of this study was to determine if varying levels of FO mixed with varying levels of linoleic acid-rich corn oil (CO) are capable of modifying the human breast carcinoma growth suppressive effect of a FO diet. In addition, we sought to determine if a relationship exists between human breast carcinoma growth and the level of lipid peroxidation products in the tumor tissue. The level of lipid peroxidation products of excised 10 g tumor composites in each group were determined by measuring 2-thiobarbituric acid reactive substances (TBARS) using a modified macro-thiobarbituric acid (TBA) assay (9).

Macro-TBA assay. Subcutaneously growing human breast carcinomas were surgically excised, placed individually in a 0.9% NaCl solution, immediately frozen (−20°C) and stored for one day. Immediately before the TBA assay, the tumors were thawed and tumor samples pooled to obtain 10-g composites. Once sufficient tumor homogenate was obtained from pooling (10-g composite), this constituted one sample. To obtain other samples, other tumors within the same group were also pooled to form 10-g samples. Three to five samples (10-g composites) were done in triplicate for each group. An antioxidant [tertiary butylhydroquinone (TBHQ); Eastman Chemical Products, Kingsport, TN], at 0.02% of total fat, as determined by a dry column extraction method (13) was added to the samples; the samples were homogenized (4°C) in a Polytron homogenizer (Ultra Turrax SDT 1810; Tekmar Co., Cincinnati, OH). The homogenates were transferred into distillation flasks containing 2.5 mL 4N HCl plus 0.5 mL silicone antifoam (Thomas Scientific Co., Swedesboro, NJ) with sufficient distilled water to yield a total volume of 100 mL/flask and distilled to collect 50 mL. Five mL of this distillate plus 5 mL of freshly prepared TBA (0.2N aqueous solution; Sigma Chemical Co., St. Louis, MO) were used for the assay. Samples were prepared in triplicate, heated in a boiling water bath for 35 min to develop the pigment, cooled and the absorbance read on a spectrophotometer (Spectronic 2000; Bausch & Lomb, Rochester, NY) at 532 nm. The absorbance values obtained were multiplied by an adjustment factor of 6.2 (14). Results were reported as μg of TBARS per g of sample.

Study 2. The experimental design of this study is provided in Table 4. The purpose and experimental design of this study was similar to Study 1, but two additional dietary groups (1% CO/19% FO and 1% CO/19% FO plus antioxidants) were added. A modified micro-TBA assay (15) was used in this study in order to individually assay each excised tumor for TBARS.

Micro-TBA assay. Subcutaneously growing human breast carcinomas were surgically excised, frozen (−20°C) for one day, thawed and individually homogenized (4°C) in the presence of an antioxidant (TBHQ, 0.02% of total fat) in a Polytron homogenizer. One mL of homogenate was combined with 2.0 mL of TBA (0.375% wt/vol), trichloroacetic acid (15.0% wt/vol) and HCl (0.25N) and mixed thoroughly. The solution was heated for 15 min in a boiling water bath. After cooling, the precipitate was removed by centrifugation (1,000 × *g* for 10 min) and the supernatant kept for analysis. The absorbance of the sample was determined at 535 nm against a blank that contains all reagents minus the homogenate. This TBA assay is a slightly modified version of the Buege and Aust assay (15). TBARS concentration of the sample was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (16) and reported as nmoles TBARS per mg of protein. The protein concentration of tumors was determined according to the method of Lowry *et al.* (17).

Study 3. The experimental design of this study is shown in the legend of Figure 1. The purpose of this study was to determine if the concentration of TBARS in individual human breast carcinomas correlated with tumor size from a single FO dietary group (1% CO:19% FO). Excised

TABLE 3

Effect of Different Ratios of Dietary Corn Oil and Fish Oil on the Growth and Lipid Peroxidation Product Concentrations of MDA-MB231 Human Breast Carcinomas in Athymic Nude Mice

Diet groups[a]	Mean mouse body weight (g) ± SE	Number of tumors	Mean tumor volume (cm^3) ± SE[b]	Mean tumor lipid peroxidation product levels (μg TBARS/g sample) ± SE[b]
20% Corn oil (12.0% linoleic acid)	28.1 ± 0.5	60	2.55 ± 0.31[c]	0.26 ± 0.04[c]
15% Corn oil: 5% fish oil (9.1% linoleic acid)	27.3 ± 0.4	64	1.23 ± 0.15[d]	0.37 ± 0.02[d]
10% Corn oil: 10% fish oil (6.2% linoleic acid)	27.1 ± 0.7	79	1.16 ± 0.61[d]	0.40 ± 0.04[d]
5% Corn oil: 15% fish oil (3.3% linoleic acid)	27.1 ± 0.3	66	0.88 ± 0.11[d]	0.59 ± 0.03[e]

[a]Twenty to twenty-five animals/group.
[b]Values with different superscript letters in the same column are significantly different at $P < 0.05$. TBARS, thiobarbituric acid reactive substances.

TABLE 4

Effect of Different Ratios of Dietary Corn Oil and Fish Oil and the Influence of Antioxidants on the Growth and Lipid Peroxidation Product Concentrations of MDA-MB231 Human Breast Carcinomas in Athymic Nude Mice

Diet groups[a]	Mean mouse body weight (g) ± SE	Number of tumors	Mean tumor volume (cm^3) ± SE[b]	Mean tumor lipid peroxidation product levels (nmol/TBARS/mg protein) ± SE[b]
20% Corn oil (12.0% linoleic acid)	30.1 ± 0.7	20	2.36 ± 0.90[c]	1.49 ± 0.04[c]
15% Corn oil: 5% fish oil (9.1% linoleic acid)	30.7 ± 1.0	25	2.08 ± 0.36[c]	1.66 ± 0.04[d]
10% Corn oil: 10% fish oil (6.2% linoleic acid)	29.9 ± 0.7	26	0.95 ± 0.14[d]	1.86 ± 0.03[e]
5% Corn oil: 15% fish oil (3.3% linoleic acid)	28.9 ± 0.6	28	0.72 ± 0.20[d]	1.96 ± 0.03[f]
1% Corn oil: 19% fish oil (0.9% linoleic acid)	28.6 ± 0.9	24	0.53 ± 0.09[d]	2.02 ± 0.07[f]
1% Corn oil: 19% fish oil + antioxidants (vitamin E 2000 IU/kg and TBHQ 2.0% total fat) (0.9% linoleic acid)	29.5 ± 0.8	19	1.65 ± 0.30[c]	1.48 ± 0.04[c]

[a]Six to nine animals/group.
[b]Values with different superscript letters in the same column are significantly different at $P < 0.05$. TBARS, thiobarbituric acid reactive substances; TBHQ, tertiary butylhydroquinone.

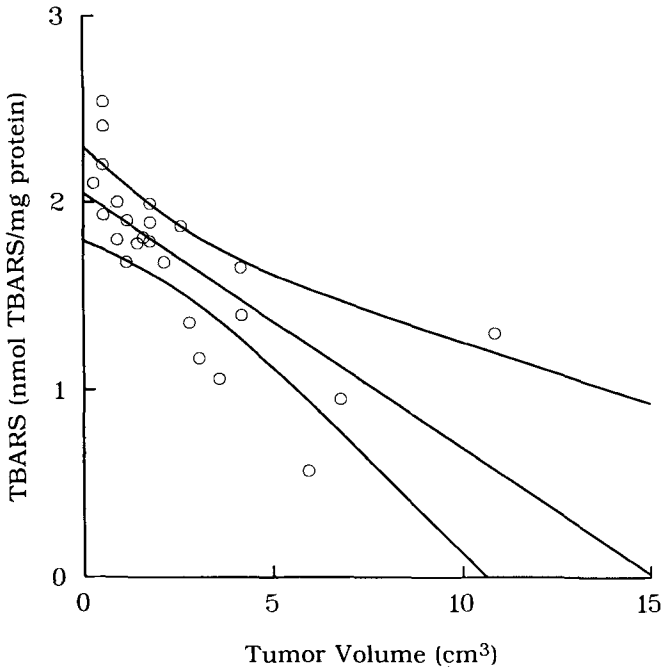

FIG. 1. Relationship between mean carcinoma volume (cm^3) and mean carcinoma thiobarbituric acid reactive substances (TBARS) concentrations per individual MDA-MB231 human breast carcinoma in athymic nude mice fed a 1% corn oil:19% fish oil diet (n = 24). There were eight animals in this dietary group. Curved lines represent a 95% confidence interval. The Pearson correlation coefficient = 0.725 ($P < 0.05$).

tumors were individually assayed for TBARS by the modified micro TBA assay.

Study 4. The experimental design of this study is provided in Table 5. The purpose of this study was to determine if the length of time the FO diet is allowed to remain in the food jar, under feeding conditions, *i.e.*, 24, 48 and 72 h after feeding, influences human breast carcinoma growth and carcinoma concentration of TBARS. The jars with diet were changed every 24, 48 or 72 h. Excised tumors were individually assayed for TBARS by the modified micro-TBA assay.

Statistical analyses. Data in Studies 1, 2 and 4 were analyzed by one-way analysis of variance and the Newman-Keuls multiple comparison test (18). Significance was set at $P < 0.05$. Data for Study 3 were examined by Pearson's correlation coefficient (18).

RESULTS

Human breast carcinoma growth. In Study 1, mean human breast carcinoma volumes (cm^3) obtained from the four dietary groups at experiment termination ranked (from largest to smallest) as follows: 20% CO > 15% CO:5% FO > 10% CO:10% FO > 5% CO:15% FO (Table 3). Mean carcinoma volumes in mice fed each of the three FO diets were significantly ($P < 0.05$) smaller than the mean carcinoma volume of mice fed CO as their sole source of fat (20% CO). Body weight gains were not significantly different among the mice in the different dietary groups.

In Study 2, mean human breast carcinoma volumes (cm^3) obtained from the six dietary groups at termination of the experiment ranked (largest to smallest) as follows: 20% CO > 15% CO:5% FO > 1% CO:19% FO plus antioxidants > 10% CO:10% FO > 5% CO:15% FO >1% CO:19% FO (Table 4). Mean carcinoma volume in mice fed 20% CO was numerically higher than those of the 15% CO:5% FO- and 1% CO:19% FO plus antioxidants-fed mice, but this difference did not reach a level of significance. However, each of these three groups had mean carcinoma volumes which were significantly ($P < 0.05$) larger than those produced by the 10% CO:10% FO, 5% CO:15% FO and 1% CO:19% FO dietary groups. Body weight gains were not significantly different among the mice in the different dietary groups.

In Study 4, mean human breast carcinoma volume (cm^3) obtained from the three dietary groups at termination of the experiment ranked (largest to smallest) as follows: 24 h, 1% CO:19% FO > 48 h, 1% CO:19% FO > 72 h, 1% CO:19% FO (Table 5). Mean carcinoma volumes in mice in which the diet (1% CO:19% FO) was changed

TABLE 5

Effect of the Length of Time the Fish Oil Diet Remained in the Food Jar Under Usual Feeding Conditions on the Growth and Lipid Peroxidation Product Concentrations of MDA-MB231 Human Breast Carcinomas in Athymic Nude Mice

Length of time diet remained in the food jar[a]	Mean mouse body weight (g) ± SE[b]	Number of tumors	Mean tumor volume (cm^3) ± SE[b]	Mean tumor lipid peroxidation product levels (nmol/TBARS/mg protein) ± SE[b]
24 h (1% corn oil: 19% fish oil)	30.6 ± 0.9[c]	24	0.70 ± 0.17[c]	2.04 ± 0.09[c]
48 h (1% corn oil: 19% fish oil)	28.5 ± 0.9	28	0.48 ± 0.11[d]	2.40 ± 0.10[d]
72 h (1% corn oil: 19% fish oil)	27.4 ± 0.5[d]	26	0.25 ± 0.27[e]	3.69 ± 0.14[e]

[a]Eight to nine animals/group.
[b]Values with different superscript letters in the same column are significantly different at $P < 0.05$. TBARS, thiobarbituric acid reactive substances.

every 24 h was significantly larger ($P < 0.05$) than in the 48-h or 72-h diet groups. The mean carcinoma volume of the 48-h diet group was significantly larger ($P < 0.05$) than that of the 72-h diet group. Body weight gains were significantly lower ($P < 0.05$) for the 72-h diet group as compared to the 24-h diet group.

Human breast carcinoma lipid peroxidation product levels (TBARS). The ranking of mean human breast carcinoma TBARS concentrations in the four dietary groups of Study 1 at termination was: 5% CO:15% FO > 10% CO:10% FO > 15% CO:5% FO > 20% CO (Table 3). Mean human breast carcinoma TBARS concentrations in mice fed each of the three FO diets were significantly ($P < 0.05$) greater than the mean carcinoma TBARS value in mice fed CO as their sole source of fat (20% CO). In Study 2, the ranking of the mean human breast carcinoma TBARS concentrations in the six dietary groups at termination was: 1% CO:19% FO > 5% CO:15% FO > 10% CO:10% FO > 15% CO:5% FO > 20% CO > 1% CO:19% FO plus antioxidants (Table 4). Mean tumor TBARS concentrations were virtually identical in the 1% CO:19% FO plus antioxidants- and 20% CO-fed animals. Mean tumor TBARS concentration in mice fed each of the four nonantioxidant-supplemented FO diets were significantly ($P < 0.05$) greater than the mean tumor TBARS concentrations in mice fed the antioxidant-supplemented FO and in the group fed only CO as their sources of fat. In Study 3, an inverse relationship is shown between volumes of individual human breast carcinomas and carcinoma TBARS concentrations in mice fed the 1% CO:19% FO diet ($P < 0.05$) (Fig. 1), *i.e.*, the smaller the tumor volumes, the greater the concentration of TBARS. In Study 4, mean carcinoma TBARS concentration in mice in which the diet (1% CO:19% FO) was changed every 24 h was significantly lower ($P < 0.05$) than in the 48- or 72-h diet groups (Table 5). The mean carcinoma TBARS of the 48-h diet group was significantly less ($P < 0.05$) than that of the 72-h diet group.

DISCUSSION

This study provides evidence that supplementation of a high-fat (CO) diet with even a modest amount of FO (*e.g.*, 15% CO/5% FO) can result in a suppression of growth of a human breast carcinoma (MDA-MB231) maintained in athymic nude mice. Other laboratories have provided evidence that FO-containing diets inhibit mammary gland tumorigenesis in rodents (1–4) and suppress growth of human breast carcinomas in athymic nude mice (5–9). Such studies often utilize diets containing FO as their only source of fat. FO is extremely low in the essential fatty acid linoleic acid (18:2). It is conceivable, therefore, that the suppression of mammary tumorigenesis exerted by diets high in FO may be *via* essential fatty acid (linoleic acid) insufficiency. Indeed, supplementation of FO diets with modest amounts of linoleic acid, in certain mammary tumor animal models, has been reported to block (4) or partially block (19) the tumorigenic inhibitory activities of a diet high in FO. In this study, we were still able to attain growth inhibition of MDA-MB231 human breast carcinomas in athymic nude mice by feeding FO diets containing substantial amounts of linoleic acid (3.3–9.1%). The estimated nutrient requirement for adequate growth and reproduction of mice for linoleic acid is 0.3% w/w of diet (20), but the level required for optimal tumorigenic processes may be substantially higher. For example, Ip *et al.* (12) have reported that approximately 4.4% of linoleic acid in the diet is required for optimal (maximal) mammary tumor development in a rodent model. In our study, diets containing FO and substantial amounts of linoleic acid (up to 9.1% of diet) still suppressed growth of this human breast carcinoma. Thus, it is likely that dietary FO-induced suppression of growth of human breast carcinoma MDA-MB231 is not due to insufficient dietary linoleic acid.

Inhibition of growth of human breast carcinoma MDA-MB231 by dietary FO may be *via* the accumulation within the tumor of cytostatic and/or cytolytic lipid peroxidation products. Evidence presented in our study which supports this conclusion is as follows. (i) As the FO content of the diet is increased, a concurrent significant increase in carcinoma TBARS concentrations is observed. (ii) Increasing the FO content of the diet decreases carcinoma growth. (ii) Those carcinomas that have the highest concentrations of TBARS have the least tumor growth. (iv) Allowing the FO diet to remain in the food jar for as long as 72 h, a feeding protocol that substantially increases the level of TBARS and the peroxide values in the diet (21), results in an increased accumulation of TBARS in the carcinomas and a concordant suppression of carcinoma growth. (v) The addition of efficacious antioxidants to the FO diet substantially decreases carcinoma TBARS concentrations and concurrently increases carcinoma growth. Collectively, these results clearly support the concept that lipid peroxidation products are relevant to the process of dietary FO-induced inhibition of breast carcinoma growth. Recent evidence from our laboratory (21) indicates that dietary FO-derived lipid peroxidation products are formed extensively in the food jar under normal feeding conditions. After feeding FO, such products are not only found in tumor tissue but are found in normal tissues as well.

The mechanism by which lipid peroxidation products retard or inhibit tumor growth processes is not certain (22). Secondary products of lipid peroxidation (measured by the TBA assay) are capable of decreasing cell proliferation through damaging cell membranes, by changing cellular composition and/or cytoskeleton assembly. These modifications in the molecular architecture of the membrane can lead to the inactivation of membrane transport systems and/or membrane bound enzymes (23–25). This phenomenon may adversely affect the entering of cells into the cell cycle or it may accelerate their exit (*i.e.*, cell death). Furthermore, secondary products of lipid peroxidation can decrease tumor cell survival by inactivating polymerase reactions (26), forming inter- and/or intramolecular linkages between amino acid sulfhydryl groups and biomolecules (DNA, RNA and proteins) (27) and inhibiting polyamine synthesis (28). Such processes may not only result in inhibition of cell proliferation but may also lead to an increase in cell death. Indeed, Garbor and Abraham (4) reported that dietary FO inhibits mammary tumor growth processes by increasing mammary tumor cell death rather than suppressing mammary tumor cell proliferation.

Although under certain conditions secondary products of lipid peroxidation may decrease tumor growth *via* a cytostatic/cytolytic mechanism, under other

circumstances such products could stimulate tumor growth. For example, a peroxidation product of linoleic acid, *i.e.*, 13-hydroxy (hydroperoxy) octadecadienoate, is more mitogenic to rat colonic mucosa cells (29) or to hamster embryo fibroblasts (30) than is the parent compound. In addition, a peroxidation product of arachidonic acid, *i.e.*, hydroxy (hydroperoxy) eicosatetraenoic acid, stimulates rat colonic mucosa cell proliferation while the parent compound itself lacks stimulatory activities (29). Thus, peroxidation products derived from fatty acids in dietary FO (particularly EPA, DHA and possibly linoleic acid) could be stimulatory to tumor growth processes. In the tumor model used in this study (MDA-MB231 human breast carcinoma maintained in athymic nude mice), however, we find no evidence of dietary FO-induced stimulation of tumor growth.

The results of our study clearly show that the addition to the FO diet of large amounts of antioxidants (vitamin E and TBHQ) significantly counteracted the inhibitory effects of dietary FO on human breast carcinoma growth. In essence, high levels of antioxidants, in the presence of dietary FO, stimulated tumor growth. Other laboratories have provided evidence that supplementation of the diet with antioxidants inhibits tumorigenesis. Clearly dietary antioxidants can suppress carcinogen activation in experimental tumor models (31). Occasionally it is reported that antioxidant supplementation can suppress tumor growth (32,33), although there is considerable evidence to the contrary (34–36). Thus, it is likely that various antioxidants can suppress genotoxic induced initiation and/or progression events in tumorigenesis; evidence that antioxidants can effectively suppress tumor growth processes, however, has not been consistent or compelling.

In conclusion, the results of this study provide evidence that dietary FO can significantly suppress the growth of MDA-MB231 human breast carcinomas maintained in athymic nude mice, even in the presence of substantial amounts of dietary linoleic acid. The suppressive effect appears to be directly related to the accumulation of TBARS in the tumor tissues.

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Higher Levels of Plasma Cholesterol Sulfate in Patients with Liver Cirrhosis and Hypercholesterolemia

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An analytical method for the determination of cholesterol sulfate (CS) in plasma using gas–liquid chromatography was developed. We measured plasma CS concentrations in patients with liver cirrhosis and hypercholesterolemia as examples of disorders that involve aberrations in cholesterol metabolism. Patients with liver cirrhosis had plasma CS concentrations that were significantly higher than those of control subjects (444.6 ± 51.7 *vs.* 253.0 ± 24.6 μg/dL, mean ± SE). The levels of other lipids were lower in cirrhotics, although the differences were not significant. There was no correlation between the levels of CS and sulfated bile acids in cirrhotic patients. CS levels in plasma were also higher in subjects with hypercholesterolemia (413.7 ± 44.5 μg/dL); however, the ratio of CS to total cholesterol (TC) clearly differed between cirrhotics and hypercholesterolemic subjects ($1.44 \pm 0.11 \times 10^{-3}$ *vs.* $3.31 \pm 0.63 \times 10^{-3}$; $P < 0.05$). Both in subjects with hypercholesterolemia and in healthy controls, the CS/TC ratio was similar and CS accounted for roughly 0.14% of the TC concentration.

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Cholesterol sulfate (CS) is a minor constituent of various mammalian tissues and fluids, including erythrocytes (1,2), spermatozoa (3), hair (4), epidermal lipid (5,6) and feces (7,8). However, little is known about the nature and quantity of CS in these sources. Only under certain conditions can CS be transformed to sulfated steroid hormones without desulfation (9). However, in healthy individuals, this transformation does not occur to any significant extent (10). The measurement of plasma CS levels is important in some clinical conditions, such as in placental or microsomal steroid sulfatase deficiency (11), and in recessive X-linked ichthyosis (12–15).

Huang *et al.* (16) have reported that substantially higher plasma CS levels are found in hypercholesterolemia. However, as far as we know, CS levels in patients with liver cirrhosis have not been reported. In cirrhotic patients, the synthesis of cholesterol is reduced and sulfation of bile acids (BA), which are the main products of cholesterol, is increased (17). We report here a reliable method for measuring the minute amounts of CS that occur in human plasma using gas–liquid chromatography (GLC) with dihydrocholesterol-3-sulfate (DHCS) as internal standard. We applied this method to determine plasma CS levels in healthy subjects as well as in patients with hypercholesterolemia and liver cirrhosis.

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Abbreviations: BA, bile acids; CS, cholesterol sulfate; DHCS, dihydrocholesterol-3-sulfate; EC, esterified cholesterol; FC, free cholesterol; GLC, gas–liquid chromatography; HBs, hepatitis B surface; HCV, hepatitis C Virus; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride; TMS, trimethylsilyl.

MATERIALS AND METHODS

Subjects. The subjects studied included 11 healthy control subjects (64.8 ± 2.6 years old; 6 male and 5 female), 13 patients with liver cirrhosis (63.4 ± 1.6 years old) and 7 age-matched subjects with primary hypercholesterolemia (64.6 ± 5.1 years old).

The cirrhotics (10 male and 3 female) included 7 cases with positive hepatitis C virus (HCV) antibody, 1 with positive hepatitis B surface (HBs) antigen, 2 with chronic alcoholism and 3 with unknown etiology. Cirrhosis was diagnosed by clinical signs (*i.e.*, spider teleangiectasia, splenomegaly and ascites) and abnormal liver tests. Diagnosis was confirmed by percutaneous liver biopsy and/or autopsy in five cases. Five of the 13 cirrhotics were in uncompensated stage. Three patients also had hepatocellular carcinoma.

In seven patients with hypercholesterolemia (two male and five female), four patients were suspected to have heterozygous familial hypercholesterolemia due to xanthelasma and Achilles tendon thickness. Other diseases or effects of medications that might have caused secondary hyperlipemia were ruled out.

Chemicals. All chemicals used were of analytical grade. Free cholesterol (FC) and DHCS were purchased from Sigma Chemical Co. (St. Louis, MO). [4-^{14}C]Cholesterol (specific activity, 51.0 mCi/mmol) was purchased from New England Nuclear Corporation (Boston, MA) and purified before use on a silica column.

Measurement of plasma CS. Fasting venous blood was obtained and stored at −20°C until assayed. Two mL of plasma was used for the analysis and 20 μg of DHCS (Sigma) was added as internal standard. The plasma was added into a mixture of 20 mL of acetone/ethanol (1:1, vol/vol) in an extraction flask, and the mixture was sonicated in an ultrasonic bath (18). The dispersion was then filtered through filter paper, and the paper was washed with 10 mL of acetone/ethanol (1:1, vol/vol). The filtrate was dried under N_2. The residue was dissolved in 4 mL of chloroform and was applied to a column of silica gel (400 mg; Gaskuro Kogyo Co., Ltd., Tokyo, Japan). The column was eluted with 8 mL of chloroform and 6 mL of ethyl acetate to completely remove FC and cholesteryl esters (19). Then CS was eluted with 6 mL of chloroform/methanol (1:1, vol/vol). The dried residue was subjected to solvolysis. After adding 3 mL of tetrahydrofuran/methanol/trifluoroacetate (900:300:1, by vol), the mixture was allowed to stand at 45°C for 2 h and was then evaporated to dryness (20). Then desulfated free cholesterol and dihydrocholesterol were dissolved in chloroform and purified by passing again through the silica column. The eluate was evaporated, and the residue was heated with trimethylsilylating (TMS) reagent (SIGMA-SIL-A, Sigma) at 60°C for 30 min. After drying under a flow of N_2, the sample was analyzed by GLC (Shimadzu GC-8A, Shimadzu, Kyoto, Japan) using flame-ionization detector and a capillary column Hi-Cap (Shimadzu) coated with cross-linked methylsilicone

(25 m × 0.32 i.d.). Oven temperature was maintained at 260°C.

The quantity of CS was calculated as followings: CS (μg) = DHCS (internal standard, 20 μg) × Ac/Ad × response factor (1.009), where Ac is the peak area of cholesterol and Ad the peak area of dihydrocholesterol; the response factor, 1.009, was derived from the detector response of a standard mixture of cholesterol and dihydrocholesterol after solvolysis and TMS derivatization.

Separation of FC from CS on the silica column was tested using the mixture of 4 mg of cold and 8 μg of ^{14}C-labeled free cholesterol in two experiments.

Plasma lipids and BA. Plasma total cholesterol (TC) and FC, as well as triglyceride (TG) levels were determined by enzymatic methods using reagent kits (Cholesterol C-test, Free Cholesterol C-test and Triglyceride G-test, respectively; Wako, Osaka, Japan). The levels of esterified cholesterol (EC) were calculated by subtracting FC from TC. High-density lipoprotein cholesterol (HDL-C) was measured enzymatically after precipitating very low density lipoprotein (VLDL) and low-density lipoprotein (LDL) with heparin and $MnCl_2$ (HDL-C test, Wako). LDL-cholesterol was calculated using Friedewald *et al.* (21) formula. Total BA levels were assayed enzymatically using 3α-hydroxysteroid dehydrogenase (Enzabile 2, Daiichikagaku, Tokyo, Japan). In patients with liver cirrhosis, sulfated BA was extracted using a Sep-Pak C_{18} cartridge (Waters Associates, Inc., Milford, MA) (22). BA levels of the extracts from the Sep-Pak were measured before and after solvolysis using the enzymatic kit. The level of sulfated BA was calculated as the difference between the values obtained before and after solvolysis (20).

Statistics. The data are expressed as a mean ± SE. To compare mean values among the three groups, analysis of variance and the least significant difference were used. A P value of < 0.05 was considered statistically significant. Correlation was examined by Spearman rank correlation coefficient analysis.

RESULTS

Plasma lipid levels. Fasting plasma lipid levels of control subjects and patients with liver cirrhosis and hypercholesterolemia are summarized in Table 1. Plasma lipid levels were higher in hypercholesterolemia and lower in cirrhotics as compared to controls. Lipid levels in the cirrhotic group were lower than those of the control group; however, no statistically significant differences were observed except for the EC/TC ratio. The total BA level was significantly elevated in cirrhotics ($P < 0.01$). The plasma TC levels were over 230 mg/dL (average 289.4 ± 29.4 mg/dL) and LDL-C levels were also elevated (198.0 ± 26.5 mg/dL) in hyperecholesterolemia.

Evaluation of the CS assay. Radioactive tracer experiment showed that FC was well separated from the CS fraction in silica column chromatography. When 2,349,380 dpm of ^{14}C-labeled FC was separated using a silica column, 72.54% (1,704,244 dpm) was eluted with 4 mL of chloroform, 29.37% (690,008 dpm) with the following 8 mL of chloroform, 0.48% (11,394 dpm) with 6 mL of ethyl acetate and 0.002% (60 dpm) with 6 mL of chloroform/methanol (1:1, vol/vol). Total recovery was 102.40%. Therefore, the contamination of the CS fraction with FC was estimated to be 2.55×10^{-3}% (values are the average of two experiments).

TABLE 1

Fasting Plasma Lipids in Control (C) Subjects and Patients with Liver Cirrhosis (LC) and Hypercholesterolemia (HC)[a]

	C (n = 11)	LC (n = 13)	HC (n = 7)
Age	64.8 ± 2.6	63.4 ± 1.6	64.5 ± 5.1
TC (mg/dL)	168.0 ± 6.7	151.5 ± 12.1	289.4 ± 29.4[b]
FC (mg/dL)	37.9 ± 1.3	48.7 ± 4.6	75.6 ± 12.5[b]
EC (mg/dL)	136.1 ± 6.7	102.8 ± 9.2	213.9 ± 18.2[b]
EC/TC (%)	77.1 ± 1.1	67.7 ± 1.9[c]	74.5 ± 1.6
HDL-C (mg/dL)	38.6 ± 3.1	36.1 ± 3.0	58.3 ± 6.8[b]
LDL-C (mg/dL)	110.8 ± 5.8	96.4 ± 10.8	198.0 ± 26.5[b]
TG (mg/dL)	105.6 ± 9.4	95.3 ± 13.9	165.4 ± 22.2[b]
Total BA (μM)	8.5 ± 3.0	30.5 ± 5.3[d]	2.9 ± 0.8

[a]Each value is the mean ± SE. Abbreviations: TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; BA, bile acid(s).
[b]Differs from LC and C groups, $P < 0.05$.
[c]Differs from HC and C groups, $P < 0.05$.
[d]Differs from HC and C groups, $P < 0.01$. However, values were not compared as the analytical methods used were different (see text).

The retention times in GLC of TMS ethers of cholesterol and of dihydrocholesterol relative to 5β-cholestane were 2.09 and 2.14, respectively (Fig. 1). Figure 2 shows the peak areas against the amounts of standard CS analyzed by GLC.

The average value of recovery of 10 μg or 20 μg of CS, which was added to a pooled sample of normal plasma, were 90.4 ± 7.7% and 100.8 ± 12.1%, respectively (n = 8). To check the reproducibility of the CS assay, eight samples from control subjects and seven samples from cirrhotics were measured in triplicate. The coefficient of variation was 10.1 ± 3.9% in controls and 14.9 ± 5.3% in cirrhotics.

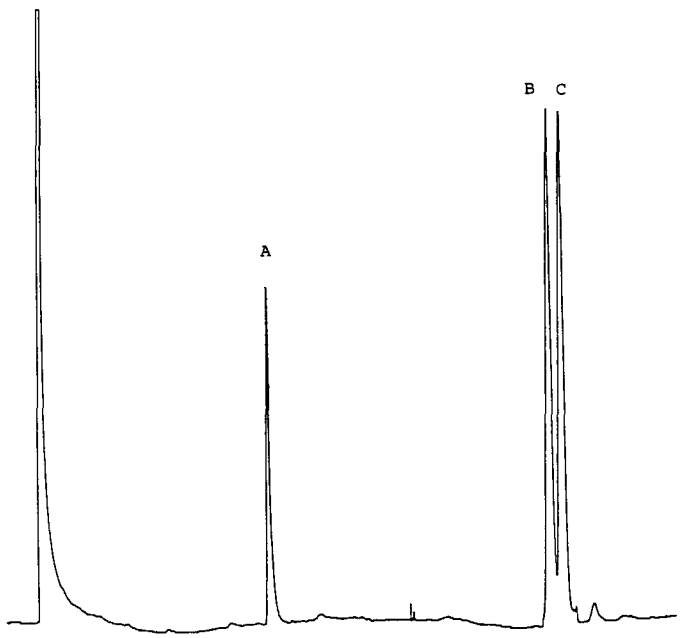

FIG. 1. Chromatographic profiles obtained from an authentic mixture of free cholesterol and dihydrocholesterol. The retention time of the trimethylsilyl ether of cholesterol (B) and dihydrocholesterol (C) relative to 5β-cholestane (A) was 2.09 and 2.14, respectively.

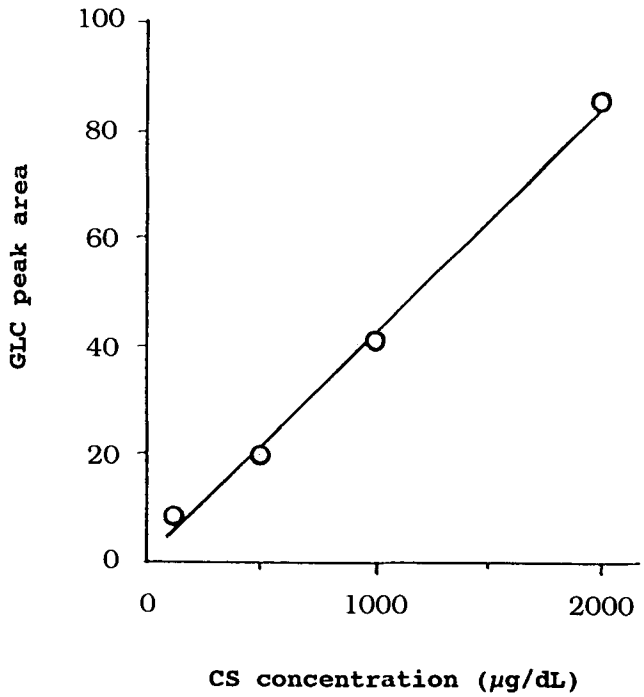

FIG. 2. Relationship between the gas–liquid chromatography (GLC) peak area and various amounts (125–2000 μg/dL) of cholesterol sulfate (CS). CS were measured as a trimethylsilyl ether of free cholesterol. A linear relationship was observed.

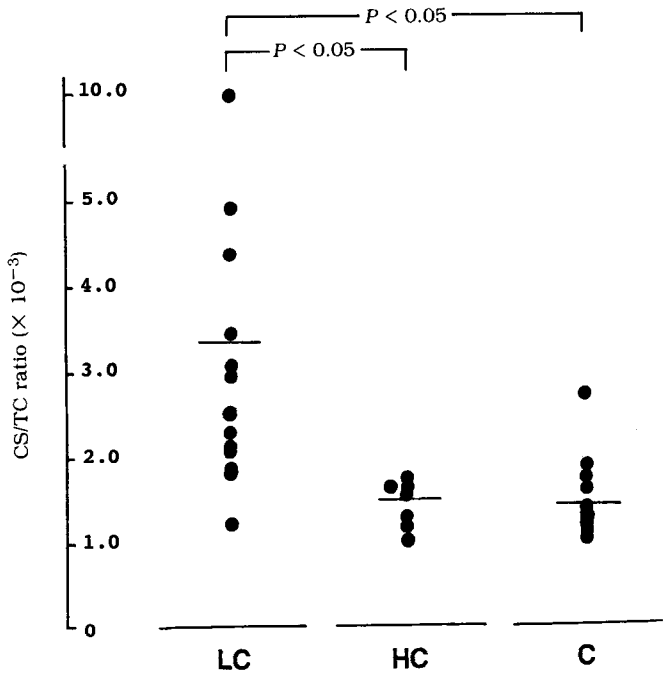

FIG. 3. The mean values of the CS/TC (cholesterol sulfate/total cholesterol) ratio in patients with liver cirrhosis (LC) and hypercholesterolemia (HC) and in control (C) subjects. The average values of the CS/TC ratio ($\times 10^{-3}$) were 3.31 ± 0.63 in the LC group, 1.44 ± 0.11 in the HC group and 1.51 ± 0.14 in the C group. The ratio in LC patients was larger than in HC or C ($P < 0.05$).

Plasma CS levels in liver cirrhosis and hypercholesterolemia. The mean values in cirrhotics and hypercholesterolemic subjects were 444.6 ± 51.7 and 413.7 ± 44.5 μg/dL, respectively. These values were significantly ($P < 0.05$) higher than the control values (253.4 ± 24.6 μg/dL).

Figure 3 shows the difference in CS/TC ratio among the three groups. The mean value of the CS/TC ratio was 3.31 ± 0.63 × 10^{-3} in cirrhotic patients, 1.44 ± 0.11 × 10^{-3} in hypercholesterolemic subjects and 1.51 ± 0.14 × 10^{-3} in control subjects. The CS/TC ratio clearly distinguished cirrhotics from hypercholesterolemics, due to the decreased plasma TC concentration in cirrhotics. In patients with uncompensated cirrhosis, CS levels (476.3 ± 117.3) were higher than those of compensated patients (424.81 ± 56.6); however, difference was not statistically significant.

Plasma BA levels were elevated in patients with liver cirrhosis (30.5 ± 5.3 μM), and sulfated BA corresponded to 22.1 ± 0.5% of total BA. There was no significant correlation between CS and total or sulfated BA. Sulfated BA levels were not assayed in control subjects and patients with hypercholesterolemia.

Plasma CS in relation to lipid levels in subjects with normal liver function. The CS/TC ratios were nearly identical in hypercholesterolemic and control subjects. When the two groups were combined as subjects with normal liver function, CS corresponded to 0.14 ± 0.09% of TC levels (n = 18). There were significant correlations ($P < 0.01$) between CS and TC (r = 0.63), EC (r = 0.67) or LDL-C (r = 0.64). However, no correlation was observed between CS and FC, HDL-C, TG or total BA.

DISCUSSION

Because our analytical procedure measures the FC liberated upon CS solvolysis, complete removal of free and acylated cholesterol from the CS fraction is an absolute requirement (23). Radioactive tracer experiments revealed that all the FC was removed from CS by elution in the silica column with chloroform and ethyl acetate.

Both CS and DHCS behaved identically throughout the various analytical steps including extraction, column chromatography and solvolysis. Thus DHCS can be used as an appropriate internal standard for the measurement of CS. Previous reports on normal human plasma CS concentrations cover a range from 105 to 350 μg/dL (14–16, 24,25) or about 0.1% of TC levels. In our analysis, the CS levels in healthy subjects were 253.4 ± 24.6 μg/dL, which is well within the range reported previously.

CS is widely distributed in the human body, including the central nervous system (23), erythrocytes (1,2), plasma (14–16,22,24,26), bile (7,8), feces (7,8), skin (5,6), nails and hair (4). Hochberg *et al.* (27) reported that liver, skin, lung and kidney could sulfurylate cholesterol. CS in plasma has been reported to bind to LDL particles (13) and chylomicrons (28). Our present results show that CS levels correlate well with LDL-C, TC and EC. This may suggest that LDL and chyromicron metabolism may be affected by an increased CS content, as an increased content of CS is known to affect the electronegativity of LDL particles (13).

The steroid sulfatase enzyme (sterol sulfate sulfohydrolase, EC 3.1.6.2) is a microsomal enzyme that removes the sulfate group from the 3-position of sterols and steroids

such as CS, dehydroepiandrosterone sulfate, pregnenolone sulfate and estrone sulfate (29). Tissues may utilize CS in sterol production by transforming CS to pregnenolone sulfate and to other 3β-hydroxy-Δ5-steroid sulfates (9,30).

Measurement of plasma CS is an important diagnostic procedure in patients with X-linked ichthyosis, because extremely elevated plasma CS levels (1500–4000 μg/dL) are observed due to steroid sulfatase deficiency (12–15). Although the increase in cirrhotics was not as pronounced as in patients with X-linked ichthyosis, we cannot rule out that decreased cholesterol sulfatase activity also occurs in cirrhotic patients. We must also consider the possibility of increased sulfation because sulfated BA increased in these patients, as they do in many other liver diseases. It has been suggested that hepatic sulfation and glucuronidation, which form more polar metabolites, enhance fecal and urinary excretion and may represent a protective mechanism in cirrhosis (17). To investigate the altered metabolism of CS in liver disease, it would also be necessary to analyze CS levels in urine and bile.

In conclusion, we have developed a method for measuring plasma CS using DHCS as internal standard. In patients with liver cirrhosis, plasma CS levels were significantly higher than those of normal subjects. The average CS/TC ratio was also significantly higher in cirrhosis due to lower TC levels. In subjects with hypercholesterolemia, plasma CS was higher but CS/TC ratios were almost the same as those in controls. In control and hypercholesterolemia subjects, plasma CS reached about 0.14% of TC and correlated with LDL-C, TC and EC, but not with FC, HDL-C and TG. The mechanism of elevated CS levels in liver cirrhosis is currently being studied in our laboratory.

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Phospholipids from the Free-Living Nematode *Caenorhabditis elegans*

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The phospholipid and the fatty chain compositions of diacyl, alkylacyl and alkenylacyl glycerophospholipids of the free-living nematode, *Caenorhabditis elegans*, were investigated. The phospholipids were comprised of 54.5% ethanolamine glycerophospholipid (EGP), 32.3% choline glycerophospholipid (CGP), 8.1% sphingomyelin and 5.1% others. The most abundant fatty acid in CGP was eicosapentaenoic acid (20:5n-3). The fatty acids in CGP were more unsaturated than those in EGP. Alkenylacyl and alkylacyl subclasses accounted for 1.0 and 2.6%, respectively, of CGP and 14.0 and 19.6%, respectively, of EGP. At least 80% of the alkenyl and alkyl groups were 18:0 chains and the remaining were odd numbered chains. The potential presence of platelet-activating factor (PAF) was examined by bioassay, but PAF-like activity was not detected in the extracts of this nematode.
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Nematodes can be divided into three major groups according to their feeding habits—plant-parasitic nematodes, animal-parasitic nematodes and free-living or microbivorous nematodes. Among the latter, *Caenorhabditis elegans* has been widely used as a model organism for the study of behavioral genetics and experimental gerontology, because these nematodes can be grown readily on a layer of bacteria and have a small, nearly constant cell number (1). Recently, the complete sequencing of the *C. elegans* genome has been initiated as a model for the human genome sequencing project (2). Using the long-lived *age-1* mutant, an attempt to biochemically explain senescence with respect to the free-radical theory was reported (3). Although *C. elegans* has been intensively studied by molecular geneticists and developmental biologists, biochemical studies on the lipids of this nematode have been limited, except for the extensive studies on its metabolism of plant sterols (4) and its fatty acid composition (5). It is known that several lower invertebrates contain remarkably high amounts of ether phospholipids, suggesting their importance in the invertebrate life cycle (6). Recently, it has also been demonstrated that the biologically active phospholipid, platelet-activating factor (PAF 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) which is metabolically derived from alkylacyl choline glycerophospholipids (CGP), is widely distributed in lower multicellular invertebrates (7). However, the available data on nematode ether phospholipids are fragmentary, with the exception of the studies of ether phospholipids from a free-living nematode, *Turbatrix aceti* (8) and a plant-parasitic nematode, *Meloidogyne javanica* (9).

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Abbreviations: CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; GC/MS, gas chromatography/mass spectrometry; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; PAF, platelet-activating factor; *t*BDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromatography; 17:Δ, *cis*-9,10-methylenehexadecanoic acid; 19:Δ, *cis*-11,12-methyleneoctadecanoic acid.

In the present study, we analyzed the phospholipids from the free-living nematode *C. elegans*, cultured on *Escherichia coli*, and also examined these lipids for the presence of the PAF molecule.

EXPERIMENTAL PROCEDURES

Chemicals. Iso-myristic acid, *anteiso*-myristic acid and *iso*-palmitic acid were from Larodane Fine Chemicals (Westbury, NY), 20:3n-9 was from Biomol (Plymouth Meeting, PA), 20:3n-6 homo-γ-linolenic acid and 20:3n-3 were from Nu-Chek-Prep (Elysian, MN), and 20:4n-6 arachidonic acid, 20:5n-3 eicosapentaenoic acid and 22:6n-3 docosahexaenoic acid were from Sigma Chemical Co. (St. Louis, MO). Glyceryl ethers (16:0 and 18:0) were purchased from Sigma, and 18:1 glyceryl ether was from Serdary Research Laboratories (London, Ontario). Phosphatidylinositol (soybean), phosphatidylserine (bovine brain), cardiolipin (*E. coli*) and phospholipase C (EC 3.1.4.3, *Bacillus cereus*) were from Sigma. Pre-coated thin-layer plates (Silica gel 60) were obtained from Merck (Darmstadt, Germany). Silic AR (100–200 mesh) was the product of Mallinckrodt (Paris, KY). Bovine serum albumin, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) and calcium ionophore A23187 were from Sigma. *tert*-Butyldimethylchlorosilane/imidazole reagent and 10% methanolic HCl reagent were from GL Sciences and Tokyo Kasei (Tokyo, Japan), respectively. Trypton and yeast extract were the products of Difco Laboratories (Detroit, MI).

Growth of E. coli. *E. coli* (OP50 strain) was grown in a phosphate buffer (pH 7.4) with trypton and yeast extract. *E. coli* from late-logarithmic cultures were isolated by centrifugation and resuspended in S medium (10). *E. coli* phospholipids were comprised of 80% phosphatidylethanolamine, 19% phosphatidylglycerol and 1% cardiolipin. No ether-linked subclasses (1-alkyl-2-acyl or 1-alk-1′-enyl-2-acyl) were detected in the ethanolamine glycerophospholipid (EGP) fraction. The fatty acid composition was 14:0, 4.8%; 15:0, 1.6%; 16:0, 41.2%; 16:1n-7, 4.0%; *cis*-9, 10-methylenehexadecanoic acid 17:Δ, 31.2%; 18:1n-7, 10.0%; and *cis*-11,12-methyleneoctadecanoic acid 19:Δ, 7.3%.

Growth of C. elegans. The standard wild-type strain N2 was propagated on NGM agar seeded with *E. coli*. Upon observation under the microscope, the plates rich with adult worms were washed with M-9 buffer (10) and samples were collected by centrifugation. Nematodes in a small volume of M-9 buffer were layered over 35% sucrose and centrifuged at 1800 × *g* for 5 min. *E. coli* and the dead worms were sedimented, and *C. elegans* was recovered on the sucrose layer and repeatedly rinsed with distilled water.

Lipid extraction and fractionation. Upon adding liquid nitrogen, frozen nematodes (from 2 to 15 g of each harvest) were thoroughly ground in a mortar with a pestle. Chloroform and methanol were added to obtain a one-phase system (11), which was sonicated for 30 min. Equal volumes of chloroform and water were added to this crude

extract, and the two phases were separated by centrifugation at 1800 × g for 5 min. The lower phase extract was then subjected to silic AR column chromatography. For 1 mg lipid-phosphorus of the extract, 1 g of silicic acid was employed. Neutral lipids were eluted with 30 mL of chloroform, and phospholipids were recovered with 30 mL of methanol. Phospholipid classes were separated by two-directional thin-layer chromatography (TLC) using the solvent systems chloroform/methanol/28% NH_4OH (65:35:5, by vol) for the first development and chloroform/acetone/methanol/acetic acid/H_2O (5:2:1:1.3:0.5, by vol) for the second development (12). For the large-scale preparation of CGP and EGP, the methanol fraction was subjected to preparative TLC using the solvent system chloroform/methanol/H_2O (65:35:6, by vol). Lipid spots were detected under ultraviolet light after spraying the plate with 0.02% 6-*p*-toluidino-2-naphthalenesulfonic acid solution. The relative amounts of CGP and EGP subclasses were determined by the successive degradation of phospholipids with mild alkali and acid, respectively (13). The phosphorus contents of the total lipids and of individual phospholipid classes were determined by the method of Bartlett (14).

Fatty chain analyses of phospholipid subclasses. Samples (1 μmol) of CGP or EGP were hydrolyzed with phospholipase C (15). Alkenylacyl, alkylacyl and diacyl subclasses of CGP and EGP were separated by TLC as 1,2-diradyl-3-acetyl-glycerols first with petroleum ether/diethyl ether/acetic acid (90:10:1, by vol) and then with toluene as developing solvents (12). The procedures for the derivatization of the fatty chains of each subclass were described previously (16). Fatty acid methyl esters were analyzed by gas chromatography (GC) (Shimadzu 14A, Kyoto, Japan) using a capillary column coated with a 0.25-μm film of polar CBP20 (0.22 mm × 30 m; Shimadzu). The temperature of the injector and flame-ionization detector was 250°C. For routine analyses, the initial column temperature was set at 170°C and then raised to 255°C at 5°C/min. For determining relative retention times, the column was maintained at a constant 200°C. Fatty acid analyses were also carried out on a Shimadzu QP-2000 quadrupole mass spectrometer equipped with an interface for capillary GC (column coated with a 0.25-μm film of nonpolar CBJ1, 0.25 mm × 30 m). The analyses were done with the injector, ion source and interface temperatures at 250°C. Samples in *n*-hexane were injected (1–2 μL) in the split mode. The oven temperature was raised from 200 to 250°C at a rate of 5°C/min for methyl esters and kept at 250°C for *tert*-butyldimethylsilyl (*t*BDMS) derivatives. Glyceryl ethers were analyzed as *t*BDMS derivatives with the oven programmed from 250 to 290°C at rate of 5°C/min. The ionization energy was 70eV.

Bioassay. Nematodes (2.24 g) isolated from 60 NGM agar plates were suspended in 20 mL of S medium, and the sample was divided into two tubes. One tube was stimulated with 1×10^{-6} M ionophore A23187, and 10 μL of vehicle, dimethylsulfoxide, was added to the other. The samples were incubated at 20°C for 15 min and then centrifuged. Lipids were extracted from the supernatant and the pellet (11), and the lipids (18 μmol phospholipid in the pellet fraction) were subjected to preparative TLC using chloroform/methanol/H_2O (65:35:6, by vol) for development. Six fractions including the PAF fraction, were suspended in an appropriate volume of 2.5 mg bovine serum albumin/mL saline solution. PAF was assessed on the basis of its ability to cause aggregation of washed rabbit platelets (17). A calibration curve was prepared for 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine ranging from 5×10^{-11} to 5×10^{-10} M.

RESULTS

Phospholipid content and composition. C. elegans was shown to contain phospholipids at a level of 187 ± 46.7 μg lipid phosphorus equivalents/gram fresh body weight based on five different samples of nematodes measured and expressed as mean ± SD. The major phospholipid constituents were EGP and CGP, accounting for 54.4 ± 4.7 and 32.3 ± 3.0% of the total phospholipids, respectively. Sphingomyelin accounted for 8.1 ± 0.7%. Other constituents were cardiolipin, inositol glycerophospholipids and lysoCGP, each of which accounted for about 1%.

Subclasses of CGP and EGP. Alkenylacyl, alkylacyl and diacyl subclass compositions of CGP and EGP are shown in Table 1. A large portion of CGP was accounted for by the diacyl subclass, whereas the alkylacyl subclass, a precursor of PAF (18), accounted for only 2.6 ± 0.6%. In contrast, EGP contained substantial amounts of alkylacyl and alkenylacyl subclasses, as well as the diacyl form.

Fatty alkyl chain composition. Alkyl and alkenyl chain compositions of 1-alkyl-2-acyl glycerophosphocholines (GPC), 1-alkyl-2-acyl glycerophosphoethanolamines (GPE) and 1-alkenyl-2-acyl GPE were determined on the di-*t*BDMS derivatives. In GC/mass spectrometry (MS), the derivatives yielded ions at *m/z* 57, 73 and 171, common to all samples, and $[alkenyl + 56]^+$ and $[alkyl + 130]^+$, indicative of each of the glyceryl ether structures.

The di-*t*BDMS derivatives obtained from 1-alkyl-2-acyl GPC were monitored on the common ion *m/z* 171 and on the individual ions $[alkyl + 130]^+$. Major species were 18:0, followed by the 19:1, 19:0, 17:0 and 18:1 alkyl moieties, whereas 16:0 alkyl was quite low (Fig. 1). Similarly, 18:0 comprised the largest percentages (89.7 and 93.8) of the alkyl and alkenyl moieties in 1-alkyl-2-acyl GPE and 1-alkenyl-2-acyl GPE, respectively. The alkyl and alkenyl compositions of CGP and EGP are shown in Table 2.

Fatty acid composition. Upon injection of the fatty acid methyl esters prepared directly from CGP and EGP, 17 peaks occurred (excluding the dimethylacetal peak due to 18:0 plasmalogen in EGP) (Table 3). These peaks were

TABLE 1

Subclass Compositions of Choline and Ethanolamine Glycerophospholipid in the Free-Living Nematode, *Caenorhabditis elegans*[a]

Subclass	CGP (%)	EGP (%)
Diacyl	96.4 ± 1.2	66.4 ± 3.7
Alkylacyl	2.6 ± 0.6	19.6 ± 2.1
Alkenylacyl	1.0 ± 0.7	14.0 ± 1.6

[a]Values are mean ± SD from four harvests of nematodes. CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids.

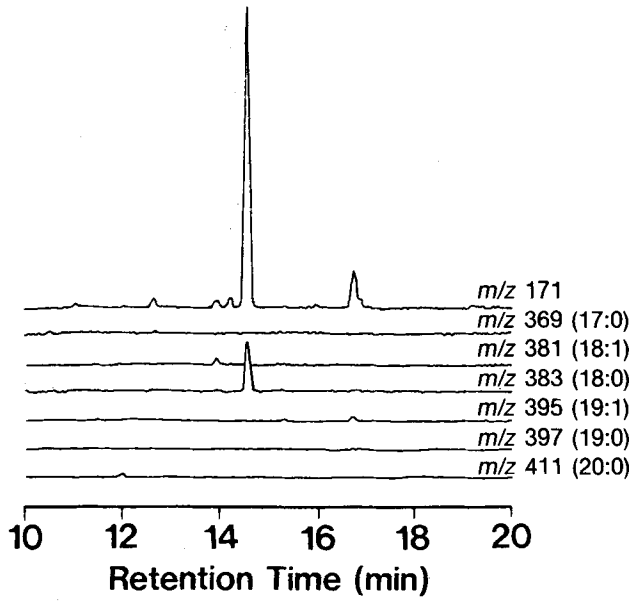

FIG. 1. Selected ion monitoring of the di-*tert*-butyldimethylsilyl derivatives of 1-alkyl glycerol from nematode choline glycerophospholipid. The alkyl chains were identified by the ions at *m/z* 396 (17:0), *m/z* 381 (18:1), *m/z* 383 (18:0), *m/z* 395 (19:1), and *m/z* 397 (19:0). The retention time of di-*tert*-butyldimethylsilyl derivative of 1-hexadecyl glycerol was 11 min 04 s. Peaks at 13 min 93 s in the tracings of ions *m/z* 171 and *m/z* 381 were due to 18:0 alkenyl.

identified as follows: (i) By comparison of their retention times with those of fatty acids from egg yolk phosphatidylcholine, peaks 3, 4, 9, 10, 12 and 15 were identified as 16:0, 16:1n-7, 18:0, 18:1n-9, 18:2n-6 and 20:4n-6. (ii) Peaks 8, 11 and 13 were identified as 17:Δ, 18:1n-7 and 19:Δ by comparison of their retention times with those of fatty acids from *E. coli* phosphatidylethanolamine. (iii) Mass spectral data demonstrated that the molecular ion of peak 1 corresponded to 15:0; peaks 2 and 3 had the same molecular weight and corresponded to 16:0; and peaks 5, 6 and 7 corresponded to 17:0. Based on relative retention time plots obtained under isothermal conditions on normal saturated fatty acids, including 12:0, 14:0, 16:0 and 18:0, branched fatty acids, *iso* 14:0, *iso* 16:0 and *anteiso* 14:0, peak 1 was identified as *iso* 15:0, peak 2 as *iso* 16:0, peak 5 as *iso* 17:0, peak 6 as *anteiso*17:0, and peak 7 as normal 17:0. (iv) Peaks 14–17 were shown to belong to a series of 20-carbon polyunsaturated fatty acids because they eluted as a single fraction upon catalytic hydrogenation over platinum oxide, the mass spectrum confirmed the C_{20} chainlength. Subsequently, the fatty acid methyl esters were saponified and then converted to their *t*BDMS derivatives, because the methyl esters of polyunsaturated

TABLE 2

Alkyl and Alkenyl Compositions of CGP and EGP from the Free-Living Nematode, *C. elegans*[a]

Chain	CGP	EGP	
	Alkyl (%)	Alkyl (%)	Alkenyl (%)
17:0	2.3 ± 0.4	2.5 ± 0.7	1.6 ± 0.2
18:1	1.8 ± 0.1	1.0 ± 0.6	—
18:0	80.4 ± 1.1	89.7 ± 0.7	93.8 ± 0.8
19:1	12.0 ± 0.1	3.7 ± 0.5	—
19:0	3.4 ± 1.6	3.1 ± 0.9	3.6 ± 0.7

[a]Ion $[alkyl + 130]^+$ or $[alkenyl + 130]^+$ was selectively monitored, and the intensities of seven ions ranging from 16:0 to 20:0, including 18:1 and 19:1, species were integrated. Values are presented as mean ± SD from three harvests; ions of less than 1% were omitted. Similar distributions were obtained by monitoring at *m/z* 171. Abbreviations as in Table 1.

TABLE 3

Fatty Acid Compositions (%) of Subclasses of CGP and EGP from Free-Living Nematode, *C. elegans*[a]

Peak no.	Fatty acid	CGP		EGP		
		Diacyl	Alkylacyl[a]	Diacyl	Alkylacyl	Alkenylacyl
1.	15:0 *iso*	1.9 ± 0.4	—	7.2 ± 3.0	1.3 ± 0.3	1.2 ± 0.2
2.	16:0 *iso*	1.4 ± 0.2	1.8 ± 0.3	—	1.2 ± 0.1	1.2 ± 0.2
3.	16:0	4.0 ± 1.5	24.7 ± 2.4	11.1 ± 0.6	4.4 ± 0.6	5.7 ± 1.2
4.	16:1 n-7	2.5 ± 0.5	10.5 ± 3.7	12.6 ± 4.0	2.8 ± 0.3	2.7 ± 1.2
5.	17:0 *iso*	2.4 ± 0.5	—	3.9 ± 1.5	1.7 ± 0.1	1.3 ± 0.1
6.	17:0 *ante*	—	—	1.5 ± 0.3	—	—
7.	17:0	1.0 ± 0.1	1.9 ± 0.1	1.6 ± 0.3	1.5 ± 0.9	—
8.	17:Δ	3.8 ± 0.7	1.1 ± 0.9	6.7 ± 0.1	2.7 ± 0.6	5.1 ± 2.6
9.	18:0	4.4 ± 0.9	8.0 ± 1.0	8.8 ± 1.7	2.4 ± 0.6	2.5 ± 0.5
10.	18:1n-9	5.0 ± 0.5	10.0 ± 2.2	2.3 ± 0.6	4.0 ± 0.7	4.6 ± 0.7
11.	18:1n-7	18.8 ± 2.4	5.0 ± 1.4	19.4 ± 2.0	33.4 ± 2.4	34.8 ± 4.4
12.	18:2n-6	5.8 ± 0.5	3.6 ± 1.0	3.8 ± 1.0	18.2 ± 1.3	8.9 ± 1.4
13.	19:Δ	4.8 ± 1.4	—	3.1 ± 0.9	1.2 ± 0.1	1.3 ± 0.1
14.	20:3n-6	6.2 ± 0.3	1.3 ± 0.9	2.8 ± 0.6	2.9 ± 0.5	4.3 ± 0.8
15.	20:4n-6	5.6 ± 0.7	1.0 ± 0.7	1.2 ± 0.2	2.1 ± 0.5	2.9 ± 0.9
16.	20:4n-3	5.5 ± 0.5	—	1.9 ± 0.5	1.8 ± 0.3	1.9 ± 0.4
17.	20:5n-3	22.0 ± 4.2	3.9 ± 1.2	5.3 ± 0.7	3.9 ± 1.1	4.2 ± 1.2

[a]Values are based on percent areas and are presented as mean ± SD from three harvests; ions of less than 1% were omitted. Other fatty acids detected in alkylacyl GPC were 14:0, 5.5%; 15:0, 3.5%; 16:0 *ante*, 1.2%; and 20:3n-9, 3.0%. Fatty acids in alkylacyl GPE were 14:0, 1.0%; and 20:3n-9, 2.1%; and in alkenylacyl GPE were 14:0, 1.3%; 16:0 *ante*, 1.2%; and 20:3n-9, 1.8%. Abbreviations as in Table 1.

[b]Because of the very low amount of alkenylacyl subclass, alkylacyl and alkenylacyl GPC were combined as ether-linked subclass.

fatty acids did not yield information on molecular weights in reliable (GC/MS). The *t*BDMS derivatives gave prominent $[M-57]^+$ ions, which were useful for molecular weight determinations (19). Selected ion monitoring focused on the ion $[M-57]^+$ at *m/z* 363 for 20:3, *m/z* 361 for 20:4, and *m/z* 359 for 20:5. Comparison of retention times with those of standards (20:3n-3, 20:3n-6, 20:3n-9, 20:4n-6, 20:5n-3) revealed that peak 14 was 20:3n-6, peak 15 was 20:4n-6, peak 15 was 20:4n-3 and peak 17 was 20:5n-3.

The fatty acid compositions of CGP and EGP are shown in Table 3. Branched, cyclopropane-ringed and polyunsaturated fatty acids were detected in the phospholipids from *C. elegans*. The major fatty acids in CGP were eicosapentaenoic acid and *cis*-vaccenic acid. Interestingly, the amount of eicosapentaenoic acid was lower in EGP, as well as in the ether-linked subclass of CGP.

Detection of PAF. PAF-like activity was not detected in the supernatant or pellet of *C. elegans*, although 18 μmol of phospholipid was purified by TLC to isolate the PAF fraction for the bioassay, which can detect a few pmol of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine and uses washed rabbit platelet. PAF activity was also not found in *C. elegans* stimulated with calcium ionophore A23187, suggesting that PAF was not present or synthesized in this nematode.

DISCUSSION

Ether-linked phospholipids are widely distributed in the animal kingdom and occur in several microorganisms, but are scarce in the plant kingdom (20). CGP and EGP are the two glycerophospholipid classes that have been analyzed extensively for the acyl composition of their ether-linked subclasses (21). General conclusions that can be drawn from these extensive analyses include the following: (i) the ether-linked subclasses of CGP and EGP usually contain a greater percentage of polyunsaturated fatty acid than do the diacyl subclasses, (ii) the subclasses of EGP often contain greater percentages of polyunsaturated fatty acids than do the corresponding subclasses from CGP and (iii) the ratio of 16:0 to 18:0 alkyl and/or alkenyl is higher in the CGP subclasses than in the EGP subclasses from the same tissue (22).

The results we obtained on the phospholipids from *C. elegans* contrast from the above general rules. *C. elegans* contains several types of fatty acids, including saturated, unsaturated (n-3, n-6, n-7 and n-9), branched and cyclopropane-ringed ones. The latter are likely to have originated from the nematodes' food, *i.e., E. coli*. This appears not to be the case for *cis*-vaccenic acid because this acid was also the major acid of *C. elegans* maintained on an aqueous medium containing heated beef liver extract (5). Moreover, CGP and at least the ether-linked EGP were synthesized by *C. elegans*, because these phospholipids do not exist in *E. coli*. CGP from *C. elegans* contained a greater percentage of polyunsaturated fatty acids than did EGP, and the alkyl and alkenyl moieties were exclusively composed of 18:0 compound. A similar situation exists in another free-living nematode, *T. aceti* (8) and in the plant-parasitic nematode, *M. javanica* (9).

PAF-like lipids are widely distributed among various lower animals, *e.g.*, 370 nmol PAF/mol total phospholipid was detected in the slug, *Incilaria bilineata*, and the alkyl chain composition of PAF agreed well with that of alkyl-acyl GPC (23). In the present study, however, PAF-like activity was not detected in *C. elegans*. Recently, Hogaboam *et al.* (24) also reported that the animal-parasitic nematode, *Nippostrongylus brasiliensis*, failed to show any secreted or worm-associated PAF-like activity.

Although PAF-like lipids occur in lower invertebrates, *e.g.*, in Annelida, Mollusca and Platylhelminthes, which contain a large amount of alkylacyl glycerophospholipids (7), nematodes appear to be quite different with respect to the molecular phospholipid species that are present, and by the absence of the phospholipid mediator PAF.

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γ-Linolenic Acid in *Anemone* spp. Seed Lipids

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γ-Linolenic acid containing oils have been found in seed lipids of a number of plants, but are restricted to certain genera and families, *e.g.*, the Boraginaceae. Some of these oils have found considerable interest for pharmaceutical and dietary use, *e.g.*, borage oil and evening primrose oil in treatment of essential fatty acid and Δ6 desaturase deficiency. Our investigation of the seed lipids of certain Mongolian and other Ranunculaceae has now shown the presence of unusual fatty acids, including considerable amounts (up to 20%) of γ-linolenic acid in certain species of *Anemone*, whereas this acid was found to be absent in other species of *Anemone*. A number of other unusual fatty acids are present in *A. rivularis* but have not yet been identified. The significance of the presence of γ-linolenic acid, a Δ6 acid, is discussed in relation to Δ5 fatty acids that had been reported to occur in the same plant family. *Lipids* 28, 841–846 (1993).

γ-Linolenic acid-containing oils are of considerable interest in the pharmaceutical industry and for use in dietary food specialties (1). In general, the distribution of γ-linolenic acid (γLn) in the plant kingdom is not well understood. γLn occurs throughout the family Boraginaceae, where it is of chemotaxonomic significance. However, it also occurs in clusters of closely related species in several other plant families (2).

The family Ranunculaceae provides us with many plant genera and species which produce seed lipids containing unusual or technically interesting fatty acids. Kaufmann and Barve (3) investigated *Aquilegia*, which still is the most prominent source (up to 70%) of columbinic acid (*E,Z,Z*-5,9,12-octadecatrienoic acid), an acid which is also found in significant amounts in many *Thalictrum* species (4,5). Other unusual fatty acids have been found in larger amounts in *Delphinium* (6), *Consolida* (7) and *Caltha* (8,9), as well as in other species of Ranunculaceae. Apart from *Caltha*, Ranunculaceae which exhibit regular flowers of radial symmetry, such as *Anemone, Adonis, Clematis* and *Ranunculus*, have been thought to contain mostly the normal set of fatty acids in their seed lipids, *i.e.*, essentially palmitic, oleic, linoleic and α-linolenic acids. Earlier research has largely confirmed this (3–10), but there is one report on the occurrence of γ-linolenic acid in *Anemone cylindrica* (11). Earle *et al.* (10) mention the occurrence of 2.6% conjugated diene in *A. coronaria* seed oil but do not give data on individual fatty acid composition. Only two reports on seed fatty acid composition of *Anemone* were found in the older literature (4,11). It was therefore of interest to determine whether *Anemone* spp. are generally capable of synthesizing γ-linolenic acid and could be used as a source of this acid.

Fatty acids in Ranunculaceae seed lipids have been reported to contain unusual Δ5 *trans* and Δ5 *cis* double bonds and chainlengths of 20 and 22 carbon atoms (3–11). It should therefore be investigated whether the early report on the occurrence of γ-linolenic acid in *A. cylindrica* (11) could be confirmed using modern analytical techniques, or if perhaps another C_{18} fatty acid with another double bond position or double bond configuration (*e.g.*, Δ5 *trans* or Δ5 *cis*) and a similar gas–liquid chromatography (GLC) retention time would be present. In addition, it was hoped that the present investigation could contribute to a better understanding of the chemotaxonomic significance of the large number of different, unusual fatty acids that have been reported to be present in various genera of the plant family Ranunculaceae.

MATERIALS AND METHODS

Seeds and seed oils. During an investigation of the local flora of Mongolia, a number of seeds from wild plant species, including *A. crinita*, were collected by a Mongolian botanical expedition. Seeds of *A. crinita* were collected in Tuv aimak, Erdene sum region, Mongolia, in August 1991. Seeds of *A. rivularis, A. nemorosa, A. ranunculoides, A. narcissiflora, A. cylindrica* and *A. altaica* were from a commercial source (Jelitto, Hamburg, Germany). Evening primrose oil was purchased in a local health shop. Cleaned seeds were ground for 10 min using a ball mill. Ground seed (0.2–5.0 g, mixed with Na_2SO_4 and sea sand) were extracted with 80 mL of hexane in a flow-through extractor. After removal of the solvent (50°C, slight vacuum), the extracts were dried under nitrogen and weighed.

Saponification and methyl ester preparation. Oil samples (200–250 mg) were saponified in 10 mL 1 N ethanolic KOH (30 min, water bath, reflux), and the unsaponifiables were removed by extraction with diethyl ether. The combined soap solutions were acidified with conc. HCl and extracted three times with 20 mL of diethyl ether each. After washing and drying, the solvent was removed under vacuum and the isolated fatty acids were weighed. The DGF BF_3 method (12) was used for the preparation of methyl esters from the fatty acids. Fatty acids (100–200 mg) were boiled under reflux on a water bath in *ca.* 5 mL 0.5 N methanolic KOH. Five mL of BF_3/MeOH was added to the mixture and boiling was continued for another 5 min. After cooling the flask, 5 mL of *n*-hexane and 20 mL of saturated aqueous NaCl solution were added and the methyl esters were extracted with *n*-hexane (3 × 20 mL). The combined hexane extracts were washed, dried and filtered, and evaporated under vacuum using a rotary evaporator and weighed.

Capillary GLC. Two gas chromatographs (Hewlett-Packard HP 5890, Palo Alto, CA) equipped with fused silica WCOT capillary columns, 50 m × 0.25 mm (Chrompack, Middleburg, The Netherlands), one with CP-SIL-88, and one with Silar 5 CP, were used for the separation and quantitation of fatty acid methyl esters. For peak area

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Abbreviations: γLn, γ-linolenic acid (18:3n-6); GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; L, linolenic acid (18:2n-6); Ln, α-linolenic acid (18:3n-3); O, oleic acid (18:1); P, palmitic acid (16:0); S, stearic acid (18:0); TG, triacylglycerol(s).

integration, a Chromato-Integrator D 2000 (Merck-Hitachi, Darmstadt, Germany) was used. Peak identification was by comparison of relative retention times with those obtained on test mixtures of known composition on two different columns.

GLC conditions. Silar 5 CP: The temperature was held at 165°C for one minute, then programmed from 165 to 205°C at 1°C/min, then held at 205°C for *ca.* 60 min before cooling down the column to 165°C. CP-SIL-88: The temperature was held at 140°C for 15 min, then programmed from 140 to 200°C at 0.7°/min. Then the column was allowed to cool to 140°C. With both columns, the detector temperature was 260°C and the injector temperature was 230°C. Flow rates were 1.13 mL N_2/min for the Silar 5 CP column and 0.86 mL N_2/min for the CP-SIL-88 column.

High-performance liquid chromatography (HPLC) of triacylglycerols. HPLC was carried out as described (13) using acetonitrile/2-propanol/hexane (70:20:10, by vol) as solvent and ultraviolet detection at 210 nm. Column was Supersphere 100 RP-18, 4 μm, 250 × 4 mm (Merck, Darmstadt, Germany).

RESULTS

Table 1 lists the species investigated here, the fat content of the seeds and the fatty acid composition of the total seed lipids after saponification and methylation. We found that some *Anemone* species contained only the usual set of fatty acids, whereas three species (*A. cylindrica, A. altaica* and *A. crinita*) contained considerable amounts (17–20%) of an acid with a gas chromatography (GC) retention time identical to that of γ-linolenic acid. A fourth species (*A. narcissiflora*) contained 6.3% of this acid. The identity of this acid as γ-linolenic acid was ascertained by co-chromatography with authentic γ-linolenic acid methyl ester on two capillary columns of different selectivity. The assignment was also confirmed by GLC/mass spectrometry and by double bond positional analysis of γ-linolenic acid from *A. crinita* and by a more detailed study of the GLC behavior of various Δ5 and Δ6 18:3 acids (14). Several short-chain saturated fatty acids (9:0, 10:0, 11:0) that were previously claimed to be present in *A. protracta* (4), were not found, and 15:0 was shown to be present in trace amounts only.

Figure 1 shows capillary gas chromatograms for three of the *Anemone* species that we have investigated for the first time, and two of which (*A. narcissiflora* and *A. altaica*) contain γ-linolenic acid.

In Figure 2, the gas chromatogram of the fatty acid methyl esters and the HPLC tracings of the triacylglycerols (TG) of Mongolian *A. crinita* seed oil are shown. Figure 3 shows comparable chromatograms for commercial evening primrose oil (*Oenothera biennis*). At first sight, the GLC tracings of *A. crinita* and *O. biennis* fatty acid methyl esters seem almost identical. The higher γLn content in *A. crinita* (19.5 *vs.* 9.7% in *Oenothera*) is, however, clearly seen in TG HPLC by the increased peaks representing TG containing two γLn residues (LγLnγLn, OγLnγLn and PγLnγLn); the different P/O (P = palmitic acid; O = oleic acid) ratio is reflected in the different TG peak area ratios of OLL/PLL and OγLnL/PγLnL (*cf.* Figs. 2 and 3, bottom).

For all the *Anemone* seed oils, the HPLC profiles of the TG are highly specific and could be used as "fingerprints." Figure 4 shows a few additional examples. The peaks were

TABLE 1

Seed Oil Fatty Acid Composition and Fat Content of Seeds of Some *Anemone* spp. from Europe, Central Asia and America, and of Evening Primrose Oil *(Oenothera biennis)* [a]

	A. crinita		*A. rivularis*		*A. nemorosa*	*A. ranunculoides*	*A. narcissiflora*	*A. cylindrica*	*A. altaica*	*Oenothera biennis* oil
Fat content (wt%)	17.8		13.1		28.0	26.7	15.0	22.7	19.5	
Fatty acid	Silar	CP-SIL	Silar	CP-SIL	Silar	Silar	Silar	Silar	Silar	CP-SIL
12:0	—	—	0.03	0.03	0.01	0.01	0.02	0.01	0.03	—
14:0	0.06	0.06	0.1	0.1	0.08	0.12	0.07	0.2	0.2	0.05
15:0	0.03	0.02	0.06	0.06	0.02	0.02	0.04	0.03	0.06	0.01
16:0	5.0	5.0	10.6	10.8	8.3	7.5	4.5	8.8	10.2	6.5
x_1	—	—	2.0	2.1	—	—	—	—	—	—
16:1n-9	0.03	0.02	0.05	0.05	0.02	0.03	0.03	0.05	0.1	0.02
16:1n-7	0.06	0.06	0.1	0.09	0.11	0.07	0.05	0.07	0.1	0.05
17:0	0.09	0.07	0.08	0.06	0.08	0.09	0.09	0.09	0.1	0.07
17:1n-7	0.03	—	0.02	—	0.03	0.03	0.03	0.02	—	—
18:0	2.2	2.2	1.4	1.4	2.2	2.1	2.1	2.8	2.2	1.9
x_2	—	—	1.0	1.0	—	—	—	—	—	—
18:1n-9	13.5	13.6	9.0	9.0	13.6	12.9	19.0	8.9	6.9	5.8
18:1n-7	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.6	0.6	0.7
18:2n-7	56.0	56.0	54.0	54.3	73.0	74.6	64.7	59.4	58.1	73.3
18:3n-6	19.5	19.8	—	—	—	0.2	6.3	17.1	19.3	9.7
18:3n-3	0.7	0.7	0.6	0.6	1.0	0.8	0.8	0.3	0.3	0.6
20:0	0.3	[b]	0.2	0.2	0.2	0.2	0.3	0.3	0.3	[b]
20:1n-9	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.2	0.1	0.2
20:2n-6	—	0.3	7.5	7.4	0.04	0.04	0.04	0.06	0.08	0.06
x_3	—	—	9.8	9.8	—	—	—	—	—	
22:0	0.2	0.2	0.2	—	0.08	0.1	0.3	0.1	0.1	0.2
22:1n-9	—	—	0.3	—	0.04	—	—	—	—	0.09
x_4	—	—	0.2	—	—	—	—	—	—	—
22:2n-6	—	—	0.3	0.3	—	—	—	—	—	—
24:0	—	0.08	0.1	0.1	0.07	0.09	0.1	0.1	0.1	0.07
Others	1.6	1.2	1.7	1.9	0.5	0.6	0.9	0.9	1.1	0.7

[a]Area-% (uncorrected) from capillary gas chromatography; x_1, x_2, x_3, x_4 are unidentified peaks present in *A. rivularis*.
[b]Overlaps with 18:3n-6 on column CP-SIL.

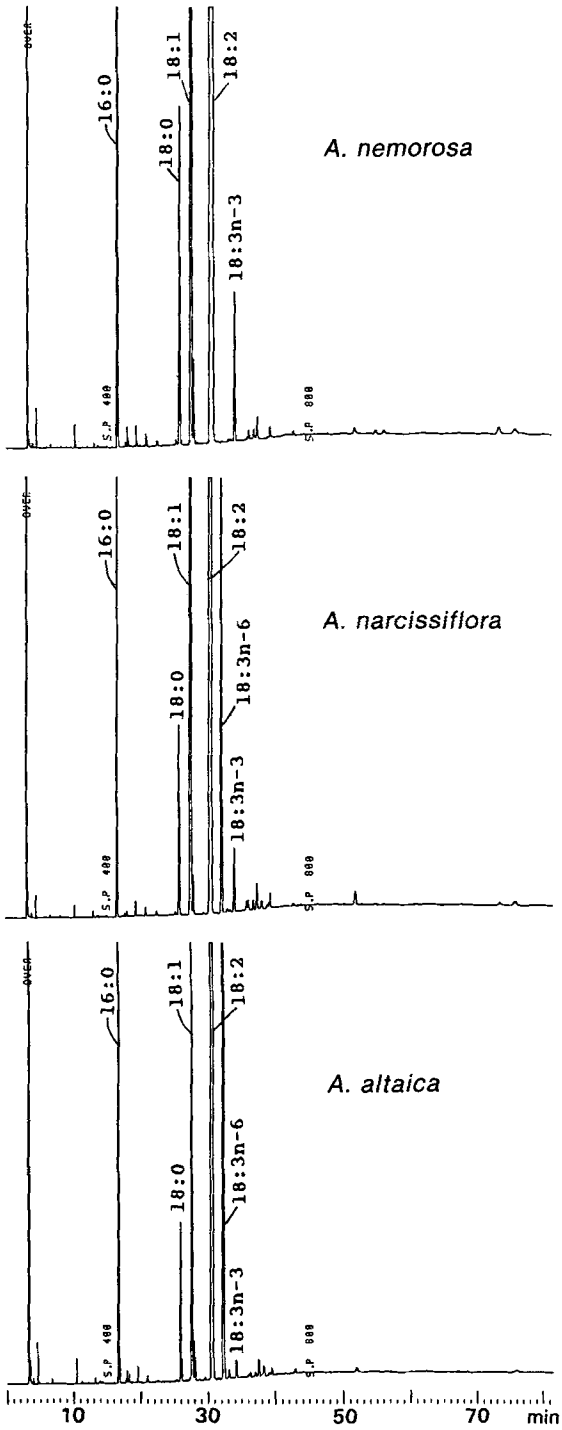


FIG. 1. Gas-liquid chromatography (GLC) of fatty acid methyl esters of *Anemone* seed lipids with and without γ-linolenic acid. The Silar 5 CP column was used; for GLC conditions, see text. Chromatograms obtained for three different species (*Anemone nemorosa, A. narcissiflora* and *A. altaica*) are shown; the fatty acid methyl ester peaks are labeled as usual.

identified in the usual way by comparison with other seed oils of known composition (13). γ-Linolenic acid-containing TG were tentatively identified by comparison with *Ribes nigrum* and *Borago officinalis* oils, and by calculating the known relative retention contribution of 18:3n-6 residues as compared with those of 18:2n-6 and 18:3n-3 residues (13). All *Anemone* oils with a high γLn content (17–20%) exhibited a large LγLnγLn peak in TG HPLC, and in this respect were similar to borage oil (13).

A totally different fatty acid pattern was found in another species, *A. rivularis*. The seed oil of this species contains two unusual fatty acids in amounts of between 7 and 10% each, but no γ-linolenic acid (Fig. 5). One of the components is 20:2n-6 (7.5%), which was also identified by co-chromatography with an authentic sample. This acid was recently found in larger quantities (up to 23%) in seeds of two *Leucojum* species (Aitzetmüller, K., unpublished data). The other major unusual fatty acid in *A. rivularis* (x_3) has not yet been identified. However, it is definitely not dihomo-γ-linolenic acid (20:3n-6). The structures of the minor fatty acids labeled x_1, x_2 and x_4 are not known.

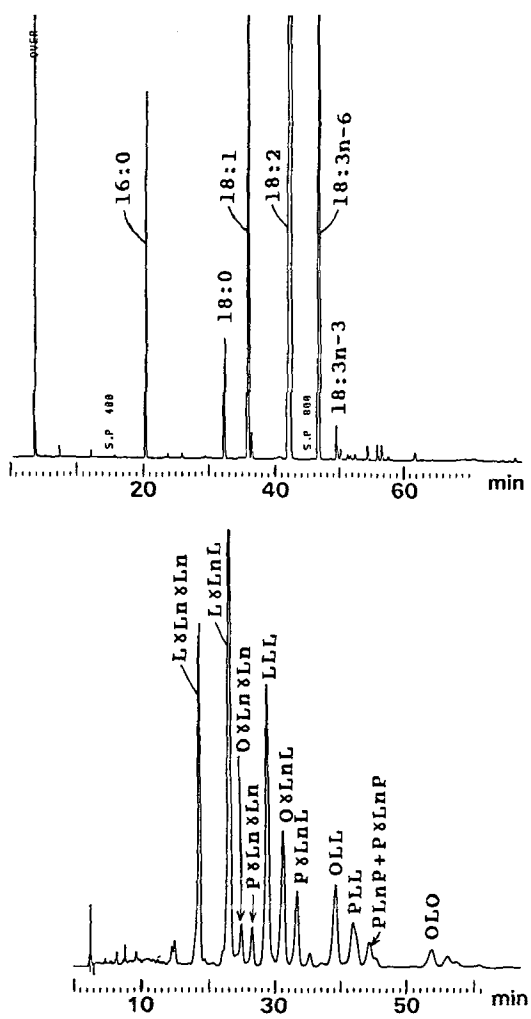
DISCUSSION

The accumulation of γ-linolenic acid in certain *Anemone* species, but not in others, could indicate a closer botanical relationship between the former species. It is possible that γ-linolenic acid is present only in the seeds of the members of one or two botanical sections within the genus *Anemone*, as it was postulated for sections of *Astelia* (15). Similarly, stearidonic acid (18:4n-3, another Δ6 acid) was recently found in one section of *Primula* (16). In the Boraginaceae, a similar case has been reported where γ-linolenic acid is completely absent in one species of the genus *Lithospermum* (in *L. officinale*), but is present in significant amounts in all other species of *Lithospermum* (17–19). Similar cases have been reported for the Liliaceae (15) and for the genus *Oenothera* (Onagraceae) (20).

The results shown here indicate a wider distribution of γLn in the plant kingdom. Apart from the Boraginaceae (17–19, 21–23), Onagraceae (20,24), Saxifragaceae (25), Aceraceae (26), Moraceae (27), Liliaceae (15), Scrophulariaceae (19) and Primulaceae (16), γLn has now been observed in at least four species belonging to the family Ranunculaceae.

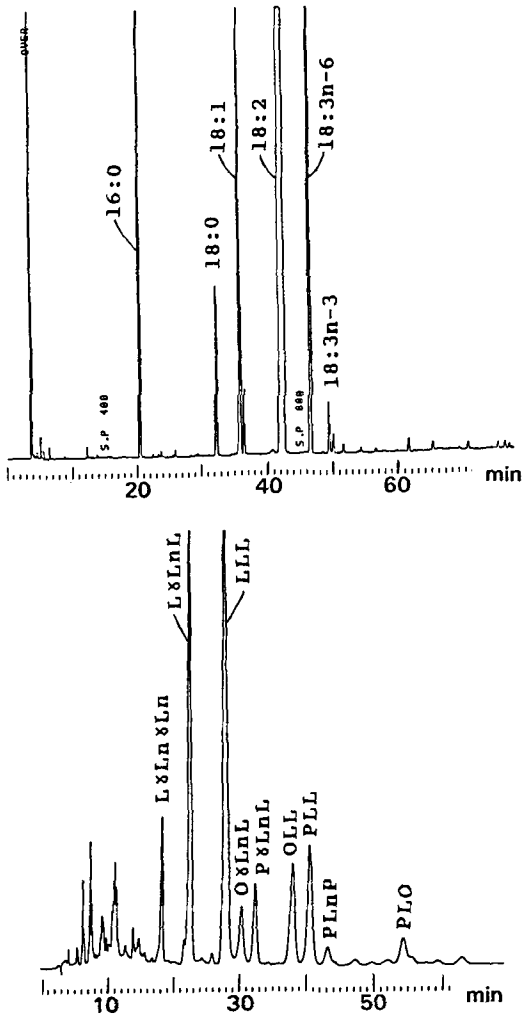
From the GC retention times, the unknown fatty acid x_3 in *A. rivularis* could perhaps be a positional or *trans* isomer of a 20:2 acid or a 20:3 fatty acid with Δ5 unsaturation [possibly the same as in *Caltha* (8)]. The same fatty acid pattern, including the unidentified fatty acids designated x_1–x_4 in Figure 5, but in lower abundance, has recently been observed for the seed lipids of another species in the Ranunculaceae, *Cimicifuga ramosa* (Aitzetmüller, K., unpublished data). The unknown peaks labeled x_1 and x_2 in Figure 5 and in Table 1 could be 16:1 Δ5 and 18:1 Δ5. Δ5-Acids of these types had been postulated to occur in the seed fats of three other genera of the family Ranunculaceae, *viz.* in *Aquilegia* (3,28), *Thalictrum* (4, 28–30) and *Caltha* (8).

Chain elongation to C_{20} acids is often observed in other genera of the Ranunculaceae [*cf.* up to 40% 20:1 in *Delphinium* and *Consolida* (6,7) and 23% 20:3 in *Caltha* (8)], but was not observed before in *Anemone*. To a lesser extent, chain elongation occurs in Boraginaceae (17–19, 21–23) where 20:1 and 22:1 (erucic) acids often accompany γLn. On the other hand, we have recently seen that chain elongation does not occur in the genus *Primula* (16), where



Anemone crinita

FIG. 2. Fatty acid [gas–liquid chromatography (GLC), top] and triacylglycerol (TG) [high-performance liquid chromatography (HPLC), bottom] chromatograms of Mongolian *Anemone crinita* seed oil. The CP-SIL-88 column was used for the GLC shown here. For GLC and HPLC conditions, see text. Fatty acid and TG peaks are labeled as usual (Ref. 13). The designation (*e.g.*, OγLnL) does not imply positional assignments.



Oenothera biennis

FIG. 3. Fatty acid (GLC, top) and triacylglycerol (HPLC, bottom) chromatograms of commercial evening primrose oil (*Oenothera biennis*). The experimental conditions were the same as for Figure 2 (GLC, CP-SIL-88 column). Abbreviations as in Figure 2.

C_{18} fatty acids (linoleic and linolenic) are further desaturated both in the Δ6 and n-3 positions to produce γLn and stearidonic acid (18:4n-3). In any case, it is interesting to note that one species (*A. rivularis*) should deviate so strongly in its seed fatty acid pattern from all other *Anemones* investigated.

From the results shown in Table 1, it seems that in two species (*A. nemorosa* and *A. ranunculoides*) fatty acid biosynthesis stops at 18:2n-6 and that this acid accumulates (up to 74% of the total), whereas in *A. altaica*, *A. crinita* and *A. cylindrica*, 18:2n-6 is further desaturated (at Δ6) to 18:3n-6. On the other hand, Δ6 desaturation does not occur in *A. rivularis*, where chain elongation successfully competes (to possibly produce 20:2n-6 from 18:2n-6). Surprisingly, 18:3n-3 is low in all species. In the 18:3 fatty acids, no evidence was found for double bonds in the Δ5 position (*cf.* Δ5,9,12-18:3 in other Ranunculaceae). Interestingly, the sum of all 18:2 plus 18:3 acids in all *Anemone* species investigated except *A. rivularis* is fairly constant (72–78%) so that an increase in 18:3n-6 seems to correlate with a corresponding decrease in 18:2n-6. This observation is consistent with the data of an earlier investigation of the seed fatty acids of *A. cylindrica* and *A. decapetala* (11). In view of the present findings, however, data on *A. protracta* published in the older literature (4) seem rather unlikely, and this species deserves to be reinvestigated. The earlier authors (4) also did not sufficiently define the term "isolinoleic" acid.

Thus, current evidence seems to suggest that with regard to seed fatty acid composition at least five different patterns or groups exist within the genus *Anemone*: Group 1: *A. nemorosa*, *A. ranunculoides*, *A. decapetala*; Group 2: *A. narcissiflora;* Group 3: *A. altaica*, *A. crinita*,

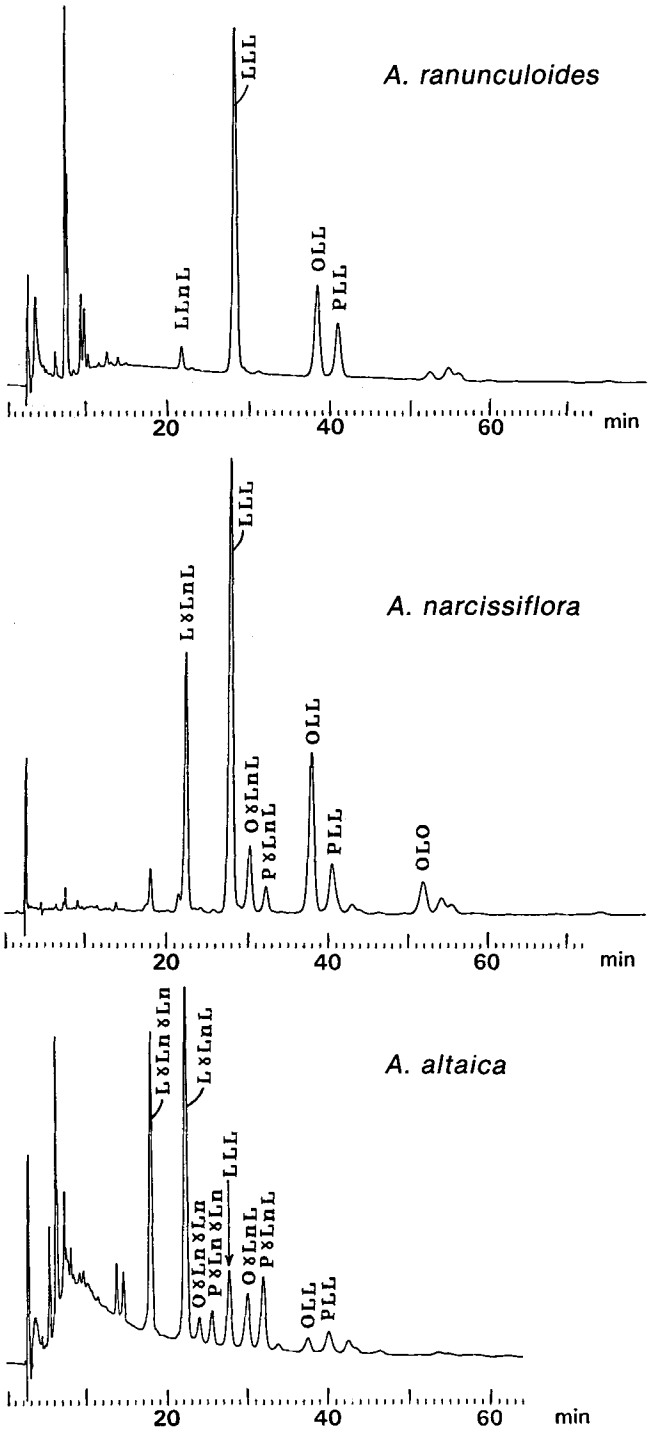


FIG. 4. HPLC chromatograms for *Anemone ranunculoides*, *A. narcissiflora* and *A. altaica* seed oil triacylglycerols. For HPLC conditions, see text. Triacylglycerol peaks, including those containing γ-linolenic acid residues, are labeled as usual (Ref. 13). The designation includes all positional isomers. Abbreviations as in Figure 2.

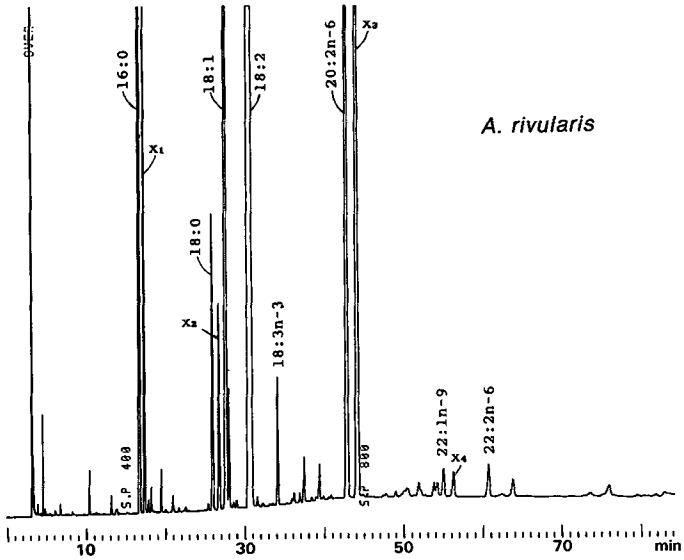


FIG. 5. Gas–liquid chromatography (GLC) of fatty acid methyl esters in *Anemone rivularis* seed oil. For GLC conditions, see text. The Silar 5CP column was used in this case (conditions as for Fig. 1). Unidentified peaks are labeled x_1–x_4, other peaks are labeled as usual.

A. cylindrica; Group 4: *A. rivularis*; and possibly Group 5: *A. protracta*. However, this is to some extent in contradiction to morphological botanical taxonomy (31), where *A. nemorosa* (2n = 30), *A. ranunculoides* (2n = 32) and *A. altaica* (2n = 16) belong to Sectio Anemonanthea; A. rivularis (2n = 16) is in Sectio Rivularidium, *A. cylindrica* and *A. decapetala* (both 2n = 16) are in Sectio Eriocephalus, whereas *A. narcissiflora* and *A. crinita* (both 2n = 14) are listed in Sectio Homalocarpus (31).

The situation in the Ranunculaceae might warrant a closer examination of the biogenetic pathways and of the enzyme systems involved in closely related species of one and the same plant family. There is Δ5 desaturation in some species [*trans* in *Aquilegia*, *cis* in *Caltha* and perhaps *trans* and *cis* in *Thalictrum* (28)], whereas we now find indication for Δ6 desaturation in several species of *Anemone*. There is chain elongation in *A. rivularis*, in *Caltha* and in *Delphinium/Consolida*. On the other hand, all three pathways are absent in *A. nemorosa* and *A. ranunculoides*. One Δ5 acid (18:3Δ5*t*,9*c*,12*c*) occurs in large quantities (30–70% of total seed fatty acids) in all the previously investigated species of *Aquilegia* and *Thalictrum*, but only in these two genera. One could pose the question whether the Δ5 18:3-producing enzyme systems in *Aquilegia* and *Thalictrum* represent a "γ-linolenic acid production gone wrong" due to a mutation of the original Δ6 desaturase? The Δ5 and Δ6 double bonds have never been observed to occur together in one species, so that the presence of isomerases seems unlikely. These relationships could become quite interesting, not only chemotaxonomically, but also for a better understanding of the biosynthesis of γLn and for biotechnological applications aiming at increased production of γLn, *e.g.*, by gene manipulation or gene transfer.

Despite the basic importance of our findings, it appears unlikely that *Anemone* will become a commercial source for γLn, because other sources, notably seeds of *Ribes*, of *Oenothera* and of *Boraginaceae*, are more readily accessible.

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Effect of Some Potassium Selective Crown Ethers on the Permeability and Structure of a Phospholipid Membrane

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The effect of some new crown ethers on the cation efflux and phase transition parameters of dipalmitoyl phosphatidylcholine liposomes was studied. The effects were correlated with the lipophilicity of the crown ethers. The results indicate that the presence of two crown ring structures in one crown ether molecule is a prerequisite for the increase of ion permeability of liposomes. The effective crown ethers decrease the temperature, enthalpy and cooperativity of the gel-to-liquid crystalline phase transition. The crown ethers increase membrane permeability for potassium and, to a lesser extent, for rubidium and sodium. The ratio of permeability increase for potassium/rubidium significantly correlates with the lipophilicity of the crown ethers.
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An important function of biological membranes is the regulation of transport of sodium and potassium ions. Certain antibiotics (valinomycin, enniatin, nigericin, monensin, X 537 A, salinomycin, narasin, etc.) show similar capabilities to differentially influence sodium and potassium transport through biological and model membranes (1-9). The effect of ionophores (nigericin and valinomycin) on the cation efflux of lipid vesicles has been studied at different temperatures. The ionophores increased the cation efflux at temperatures above the pretransition temperature, and the effect prevailed above the main transition temperature (10). Because crown ethers also exhibit marked ion selectivity (11-16), it was expected that they would selectively influence the efflux of ions through biological and model membranes. However, to increase membrane permeability, the crown ethers have to be incorporated into the membrane, and this requires special structural characteristics and optimal lipophilicity of the molecule. The objectives of our study were to determine the effect of new crown ethers on the permeability and melting properties of dipalmitoyl phosphatidylcholine (DPPC) liposomes and to assess the effect of the lipophilicity of crown ethers on their membrane-modifying properties. The correlation between structural and functional changes is similar in the bilayers of biological and model membranes (17). Therefore, hydrated DPPC appears to be a suitable model to study the effects of membrane modifying agents (18-23).

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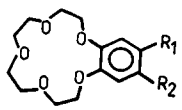
Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; ΔH_m, enthalpy of the main transition; r, coefficient of correlation; $r_{95-99\%}$, tabulated value of r corresponding to 95 and 99% significance level; R_M, lipophilicity; s_{b1-2}, standard deviations of slopes 1 and 2; S, ion selectivity; $\Delta T_{1/2}$, half-width of the main transition; T_m, main transition temperature; T_p, pretransition temperature; $t_{95\%}$, tabulated t value corresponding to 95% significance level.

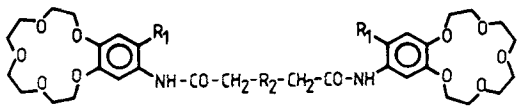
MATERIALS AND METHODS

DPPC, purchased from Sigma Chemical Co. (St. Louis, MO), was used without further purification. The crown ethers were prepared at the Department of Organic Chemical Technology of the Technical University of Budapest by Agai and co-workers (24,25). The chemical structures of the crown ethers used in this study are shown in Schemes 1 and 2. For the preparation of the phospholipid



	R_1	R_2
1	H	$-OC_2H_4OC_2H_4N(C_2H_5)_2$
2	H	$-OC_2H_4-N$ O
3	H	$-NHC_2H_4-N$ O
4	NO_2	$-NH-CONH-C_{16}H_{33}$
5	H	$-NH-CONH-C_3H_6-N$ O

SCHEME 1



	R_1	R_2
6	H	$-CH_2-S-CH_2-$
7	NO_2	$-CH_2-S-CH_2-$
8	H	$-C_3H_6-$
9	NO_2	$-C(C_2H_5)(C_2H_5)-$
10	NO_2	$-C(C_4H_9)(C_4H_9)-$
11	NO_2	$-C(CH_3)(C_{12}H_{25})-$

SCHEME 2

vesicles, 100 mg of DPPC was dispersed in 5 mL of 0.16 M KCl, NaCl or RbCl solution (containing 2 MBq of ^{42}KCl, $^{24}NaCl$ or $^{86}RbCl$) by mechanical shaking for 10 min. The lipid dispersion was then sonicated for 20 min by placing the tube in a small bath treatment vessel of the sonicator (Measuring Scientific Equipment Ltd., Manor Royal, Crawley, Sussex, England; Model 150 watt Ultrasonic Disintegrator, operating frequency, 20 kHz) working under a nitrogen stream. The bath temperature was maintained at 42°C by circulating water. After sonication, the dispersion was almost clear and the ultraviolet spectrum did not indicate oxidation. Deacylation products of DPPC were not detected by thin-layer chromatography, which was performed as described (26).

Determination of liposome permeability. After overnight equilibration at room temperature, the lipid dispersion was passed through a column of Sephadex G-50 Superfine (Pharmacia Fine Chemicals, Uppsala, Sweden) (1.5 × 30 cm) to remove excess tracers not trapped within the liposomes. The use of overnight equilibration was motivated by the fact that 8–10 h minimum are needed to reach the equilibrium of isotopes between the outer and the inner phase of liposomes (27). The liposomes were eluted from the column with 0.16 M KCl, NaCl or RbCl solutions, respectively (flow rate 0.5 mL/min). The liposome peak, which was eluted with the void volume, was collected. The initial ^{42}K, ^{24}Na or ^{86}Rb content of the liposomes (N) was determined in a gamma scintillation counter. The crown ethers were dissolved in chloroform, making a stock solution (the concentration of the stock solutions varied between 0.5–1.5 mg crown ether/mL chloroform depending on the molecular weight of the crown ether). An aliquot (0.05 mL) of stock solution was pipetted into a tube and the chloroform was evaporated at room temperature under a flow of air. Three-mL portions of eluted liposomes were added and the tube was shaken thoroughly. The crown ether/DPPC molar ratio was held constant at 1:100. Liposomes without crown ethers served as controls. The 3-mL portions (V_1) were dialyzed against 10 mL of 0.1 M KCl, NaCl or RbCl solution (V_2), respectively, at 38°C.

We are well aware of the fact that at temperatures below the gel-to-liquid crystalline phase transition temperature, there is an increased likelihood for ion leakage through a bilayer in the gel phase. However, since it has been previously established that ionophores can also increase the cation efflux between the pretransition and main transition temperatures (10) and because the experimental temperature (38°C) used in this study was above the pretransition temperature of DPPC, we made the reasonable assumption that the permeability changes observed reflected the changes caused by the crown ethers and were not due to nonspecific leakage through the DPPC bilayer.

The dialysis solutions were changed every 15 min for 2 h. The amount of radioactive tracer (n_2) in each dialysate was also determined in a scintillation counter. (The amount of lipid was determined in separate experiments by the quantitative determination of the inorganic phosphate content of the samples after $HClO_4$ treatment.) In order to compare the effects of crown ether derivatives to those of valinomycin on the ion permeability of liposomes, valinomycin was used at a molar ratio of 1:200, because at the molar ratio of 1:100, valinomycin disorganized the DPPC liposomes. Each experiment was run in quadruplicate. The permeability time constants were calculated according to the method of Johnson and Bangham (28):

$$P = \ln[V_2/(V_1 + V_2) - n_2/N] \cdot 1/t \quad [1]$$

where P is the permeability time constant, V_1 the volume of the liposome solution, V_2 the volume of dialysing solution, n_2 the amount of radioactive tracer in the dialysate, N the initial content of radioactive tracer of the liposomes and t the dialysis time in minutes. The degree of permeability increase caused by the crown ethers was calculated according to Equation 2 (ΔP):

$$\Delta P = (P - P_0) \cdot 10^{-4} \cdot s^{-1} \quad [2]$$

where P is the permeability time constant of DPPC liposomes determined in the presence of crown ethers and P_0 is the permeability time constant of DPPC liposomes determined in the absence of crown ethers.

To assess the selectivity differences among the crown ether derivatives, linear correlations were calculated between the ΔP_K, ΔP_{Rb} and ΔP_{Na} values for each compound:

$$y = a + b \cdot x \quad [3]$$

where y and x are various ΔP values determined for K^+ (ΔP_K), Rb^+ (ΔP_{Rb}) and Na^+ (ΔP_{Na}). When the equation shows a high correlation (high value for the coefficient of correlation), the ion selectivities are similar. The intercepts of linear correlations are related to the average difference of ΔP values of the compounds.

Ion selectivity (S) was defined as the ratio of the permeability increase of various monovalent cations caused by crown ethers:

$$S_{K^+/Rb^+} = \Delta P_K/\Delta P_{Rb} \quad [4]$$

$$S_{K^+/Na^+} = \Delta P_K/\Delta P_{Na} \quad [5]$$

where S_{K^+/Rb^+} is the K − Rb selectivity of crown ethers under the experimental conditions, S_{K^+/Na^+} is the K − Na selectivity of crown ethers under the experimental conditions and ΔP_K, ΔP_{Na} and ΔP_{Rb} are the permeability increases caused by crown ether incorporation.

Determination of lipophilicity of crown ethers. The lipophilicity of crown ethers was determined by reversed-phase thin-layer chromatography (29,30). Silufol UV_{254} plates (Kavalier, Brno, Czechoslovakia) were impregnated by overnight prerunning in a solution of 5% paraffin oil in *n*-hexane. The crown ethers were dissolved in dimethylformamide at a concentration of 1 mg/mL, and 5 μL of each solution was spotted onto the plates. As the crown ethers exert their effect in an ionic environment and the ions considerably modify the lipophilicity of bioactive compounds (31), the lipophilicity of crown ethers was determined in ion-free (water/acetone, 3:7, vol/vol) and in ion-containing eluents (water/acetone, 3:7, vol/vol; NaCl added to a 0.23 M final concentration). After developing, the plates were dried at 105°C and the fractions were detected by their absorption spectra in visible and ultraviolet light. Five independent developments were carried out for each lipophilicity determination.

The lipohilicity values (R_M) were calculated from the measured R_f values according to Equation 6 (29,30):

$$R_M = \log (1/R_f - 1) \quad [6]$$

The lipophilicity values determined under ionic and ion-free conditions were correlated with the membrane damaging effect (ΔP) and selectivity (S):

$$\Delta P_{K^+} = a + b_1 \cdot R_M + b_2 \cdot R_M^2 \quad [7]$$

$$S_{K^+/Rb^+} = a + b_1 \cdot R_M + b_2 \cdot R_M^2 \quad [8]$$

Determination of the melting properties of DPPC. DPPC and the crown ethers were dissolved in chloroform in the molar ratio of 100:1, and then the solvent was evaporated under a stream of nitrogen at room temperature. Twice-distilled water was added to the sample in a weight ratio of water/DPPC (4:1). The samples were vigorously mixed for 30 min above the phase transition temperature of DPPC in a Vortex mixer. The measurements were carried out on a DuPont Thermoanalyzer 990 (Barley Mill, Wilmington, DE) at a heating rate of 5°C/min and at a sensitivity range of 0.1-0.2 milliwatt/cm.

The equipment was calibrated using indium. High heating rates were required because the pretransition temperatures were not detectable at lower heating rates due to the limited sensitivity of the calorimeter. In order to prove that the differences between the half widths of the main transition of the various samples are not an artifact of the 5°C/min heating rate, the differential scanning calorimetry behavior of pure DPPC and DPPC with compound 11 mixtures were determined at 0.5, 1, 2 and 5°C/min heating rates, and the half widths were plotted against the heating rate. Three parallel determinations were carried out for each DPPC/crown ether mixture. The pretransition temperature (T_p) and main transition temperature (T_m), the half width of the main transition ($\Delta T_{1/2}$) related to the cooperativity, and the enthalpy of the main transition (ΔH_m) were calculated.

RESULTS AND DISCUSSION

The lipophilicity (R_M), the increase of permeability of liposomes caused by crown ethers, and their ion selectivity are compiled in Tables 1 and 2. The permeability time constants of the control were 0.3×10^{-4} s^{-1} for K^+ and Rb^+ and 0.5×10^{-4} s^{-1} for Na^+. Compounds containing two crown rings in the molecule showed a considerable permeability increase which was highest for potassium, followed by rubidium and sodium. This indicates that the effect of these compounds is commensurate with that of valinomycin (Fig. 1). Significant linear correlations were found between the potassium/rubidium and potassium/sodium permeabilities (Table 3). The calculated coefficient of correlation values were higher than the corresponding tabulated ones: $r_{99\%} = 0.6851$ for K^+/Rb^+ and $r_{95\%} = 0.8114$ for K^+/Na^+. These findings support the idea that the permeability increase exerted by crown ethers consists of at least two components: (i) their ability to disarrange the membrane structure and (ii) their ability to selectively increase potassium efflux.

As the permeabilities for different monovalent cations correlate well with each other, factor (i) has to be independent of selectivity. Factor (ii) is assumed to account for the selectivity: P_K values are generally higher than P_{Rb} values.

The ability of crown ethers to increase the potassium permeability of DPPC liposomes was independent of their lipophilicity. The F values of Equation 6 were 2.15 and 2.05 for R_M values determined in ion-free and ion-containing eluents, respectively.

TABLE 1

Lipophilicity (R_M) of Crown Ethers Determined in Ion-Free and Ion-Containing Environments

Compound number	R_M	
	Ion-free environment	Ion-containing environment
1	1.51	1.51
2	−0.20	−0.90
3	0.76	0.76
4	0.99	0.81
5	1.09	1.55
6	−0.28	−0.79
7	−0.23	−0.50
8	−0.39	−0.78
9	0.06	0.08
10	0.30	0.33
11	0.64	0.50

TABLE 2

Increase in Ion Permeability of Liposomes Caused by Crown Ethers

Compound number	$\Delta P \cdot 10^{-4} sec^{-1}$			S_{K^+/Rb^+}	S_{K^+/Na^+}
	K^+	Rb^+	Na^+		
1	2.23 ± 0.11	1.14 ± 0.06	0.51 ± 0.04	1.96	4.37
2	0.28 ± 0.03	0.19 ± 0.04	n.d.[a]	1.47	n.d.
3	0.31 ± 0.05	0.00	0.54 ± 0.03	∞	0.57
4	0.54 ± 0.06	0.22 ± 0.03	n.d.	2.46	n.d.
5	0.33 ± 0.04	0.34 ± 0.05	n.d.	0.97	n.d.
6	12.20 ± 0.15	7.95 ± 0.10	n.d.	1.53	n.d.
7	5.58 ± 0.08	4.14 ± 0.08	0.50 ± 0.04	1.35	11.16
8	6.87 ± 0.10	5.21 ± 0.11	n.d.	1.32	n.d.
9	11.64 ± 0.18	4.15 ± 0.09	0.90 ± 0.05	2.80	12.93
10	8.35 ± 0.14	1.83 ± 0.07	0.70 ± 0.04	4.56	11.93
11	8.75 ± 0.11	1.48 ± 0.06	0.79 ± 0.05	5.91	11.08

[a]n.d., Not determined.

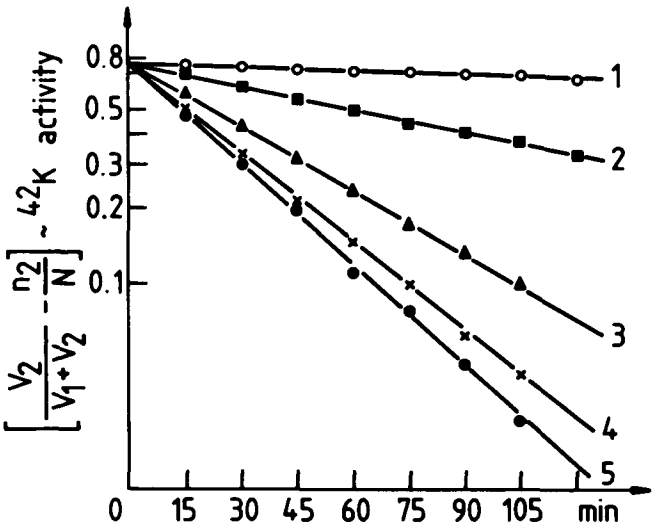


FIG. 1. The ^{42}K content of liposomes plotted as a function of time. The crown ethers and valinomycin were added to the liposomes in the molar ratio of 1:100 and 1:200, respectively. 1, Dipalmitoyl phosphatidycholine (DPPC); 2, DPPC + compound 11; 3, DPPC + compound 9; 4, DPPC + valinomycin; 5, DPPC + compound 6.

TABLE 3

Parameters of Linear Correlation Between the Increases of Permeability of Liposomes (ΔP) Caused by Crown Ethers Determined in Presence of ^{24}Na, ^{42}K and ^{86}Rb

		y = a + b • x			
y	x	n	a	b	r
ΔP_K[a]	ΔP_{Rb}[b]	11	1.44	1.69	0.8006
ΔP_K[a]	Δ_{Na}[c]	6	22.55	−8.66	0.8771
ΔP_{Rb}[b]	ΔP_{Na}[c]	6	3.40	−0.11	0.3362

[a]ΔP_K, permeability increase determined for K^+.
[b]ΔP_{Rb}, permeability increase determined for Rb^+.
[c]ΔP_{Na}, permeability increase determined for Na^+.

TABLE 4

Parameters of Quadratic Correlation Between the Potassium − Rubidium Selectivity (S_{K^+/Rb^+})[a] and Lipophilicity (R_M) of Some Crown Ether Derivatives[b]

	$S_{K^+/Rb^+} = a + b_1 \cdot R_M + b_2 \cdot R_{M^2}$	
	Ion-free environment	Ionic environment
n	10	10
b_1	3.85	1.47
b_2	−3.43	−1.90
a	2.90	3.69
r^2	0.5095	0.6294
b_1, %	50.31	40.17
b_2, %	46.91	59.83
s	1.28	1.11
s_{b1}	1.46	0.52
s_{b2}	1.31	0.57
F	3.64	5.94

[a]Calculated from the permeability increase of liposomes caused by crown ethers.
[b]Determined by reversed-phase thin-layer chromatography. Abbreviations: a, intercept, b_1, b_2, slope; b_1,%, b_2, %, normalized slope; r^2, ratio of the dependent variable determined by the change of independent variable; s, SD; s_{b1}, s_{b2}, SD of slopes 1 and 2; F, calculated value of the F test.

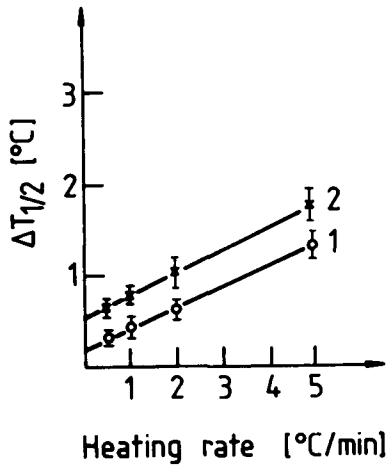


FIG. 2. Effect of heating rate on peak half-widths of DPPC (line 1) and DPPC/compound 11 (line 2) mixtures. Abbreviation as in Figure 1. $\Delta T_{1/2}$, half-width of the main transition.

The potassium/rubidium selectivity (S_{K^+}/S_{Rb^+}) correlated well with the lipophilicity values (Table 4), and the correlation was better for the R_M values determined in an ionic environment. The data suggest that not only the steric parameters, such as dimensions of the crown ring and those of the substituents, but also the lipophilicity, considerably affects the selectivity. The results indicate that the perturbation induced in the membrane bilayers by the crown ethers was related to the lipid solubility of the crown ethers. However, the change in lipophilicity accounts for only about half of the change in selectivity (r^2 values are 0.51 and 0.63 for the ion-free and ionic environment, respectively), which means that other parameters seem to influence the selectivity as well.

The peak half-width of main transition temperature of DPPC and DPPC/compound 11 mixtures decreased linearly with decreasing heating rate (Fig. 2). However, the peak half-widths of DPPC and DPPC/compound 11 mixtures significantly differed even at the lowest heating rate showing that the differences observed between the peak half-widths are not artefacts of the high heating rate. The effect of crown ethers on the melting properties of DPPC are compiled in Table 5. The pretransition and the main transition temperatures of DPPC agree well with the corresponding data in the literature (32–34); however, the ΔH_m is higher than the accepted value (33,34). This discrepancy may be due to the low sensitivity of the calorimeter; however, we believe that the differences observed between the enthalpies of the main transition temperatures reflect the real differences between the melting properties of the samples.

The crown ethers with one ring structure in the molecule have a negligible effect on the main transition temperature of DPPC/water dispersions. The *bis*-crown ethers with two ring structures per molecule decrease the ΔH_m, the T_p and main transition (T_m) temperatures, and broaden the temperature interval of the main transition ($\Delta T_{1/2}$). These data suggest interactions between the *bis*-crown ethers and DPPC. As a consequence, the crown ethers decrease

TABLE 5

Effect of Crown Ether Derivatives on the Pretransition Temperature (T_p) and Main Transition Temperature (T_m), the Enthalpy (ΔH_m) and Half-Width ($\Delta T_{1/2}$) of Main Transition of a DPPC/Water Dispersion[a]

Compound number	T_p (°C)	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH_m (mJ/mg)
Control (DPPC)	35.5 ± 0.7	41.5 ± 0.3	1.3 ± 0.1	56.0 ± 2.2
6	35.2 ± 0.9	40.5 ± 0.4	1.5 ± 0.3	45.3 ± 3.5
7	35.3 ± 1.1	40.6 ± 0.6	1.5 ± 0.3	53.8 ± 4.1
8	34.9 ± 0.7	40.3 ± 0.3	1.6 ± 0.2	46.3 ± 6.4
9	32.2 ± 0.8	40.2 ± 0.5	1.5 ± 0.3	51.0 ± 2.6
10	disappeared	40.1 ± 0.7	1.7 ± 0.4	40.3 ± 2.8
11	31.5 ± 1.0	40.0 ± 0.6	1.7 ± 0.2	50.0 ± 3.2

[a]Dipalmitoyl phosphatidylcholine (DPPC)/crown ether molar ratio 100:1. Compounds 1-5 had no effect on the thermal parameters.

the lateral order of the bilayer and the phase transition temperature.

In summary, we conclude that the type of crown ethers studied modify the structure of the lipid bilayers and increase membrane permeability. The effects depend on the lipophilicity and on the structural characteristics (mono- or *bis*-crown) of the crown ether derivatives.

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Synthesis of Ethyl Arachidonate-19,19,20,20-d_4 and Ethyl Dihomo-γ-linolenate-19,19,20,20-d_4

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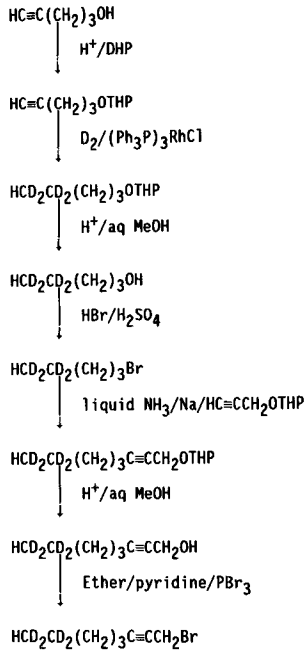
Ethyl 5,8,11,14-eicosatetraenoate-19,19,20,20-d_4 and ethyl 8,11,14-eicosatrienoate-19,19,20,20-d_4 were synthesized by Grignard coupling of the methanesulfonyl ester of 2,5-undecadiyn-1-ol-10,10,11,11-d_4 with 5,8-nonadiynoic acid and 8-nonynoic acid, respectively. The coupled products upon Lindlar reduction, followed by the preparation of their ethyl esters, yielded deuteriated ethyl arachidonate and ethyl dihomo-γ-linolenate, which were completely characterized by ^{13}C and ^1H nuclear magnetic resonance and mass spectral analysis.
Lipids 28, 853–856 (1993).

Deuterium-labeled arachidonic acid is frequently used as an internal standard to quantify the amount of arachidonic acid released from membrane lipids in response to agonists (1). Deuteriated arachidonic acid also serves as the precursor for the biosynthesis of eicosanoids (2). Reduction of 5,8,11, 14-eicosatetraynoic acid by deuterium gas in the presence of Lindlar's catalyst yields arachidonic acid labeled with deuterium at the double bonds. These preparations generally are a mixture of about 85% octadeuteriated and 15% hepta- and hexadeuterio-arachidonic acid with no unlabeled compound (2–4). The synthesis of arachidonic acid labeled with deuterium atoms at the double bonds suffers in that a high isotopic purity is not always obtained (2–4). When these preparations of arachidonic acid are used to synthesize eicosanoids the isotopic composition of the resulting compounds may be altered further (3).

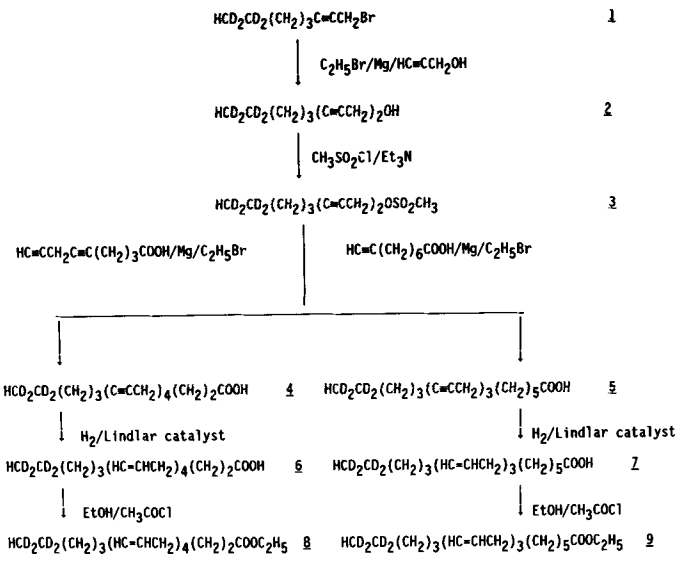
The β-oxidation of fatty acids results in the repeated loss of acetate units from the carboxyl end of the molecule. Numerous inborn errors of fatty acid oxidation have been described which result in the accumulation of chain-shortened intermediates (5). Recent studies have shown that the removal of the double bond at position 5, during unsaturated fatty acid oxidation, proceeds *via* a novel sequence of enzymatic reactions (6,7). Acids labeled with deuterium at or near the methyl terminus of the fatty acid would be of considerable value as the deuterium would not be isomerized when the acid is used as a substrate for eicosanoid biosynthesis. Secondly, these acids would be valuable probes for determining what metabolites accumulate during fatty acid oxidation in that the label would be retained during the entire β-oxidation spiral. Emken, Rakoff and their colleagues (8–13) have made extensive use of Wittig chemistry to introduce deuterium on internal carbons in fatty acids. In this study we describe, for the first time, the synthesis of deuteriated ethyl arachidonate and ethyl dihomo-γ-linolenate containing deuterium at carbon atoms 19 and 20.

RESULTS AND DISCUSSION

A primary objective of this study was to synthesize a common deuteriated precursor, in good yield, which could then be used to prepare both title compounds. The pathway for synthesis of 1-bromo-2-octyne-7,7,8,8-d_4 (1) (Scheme 1) utilizes a series of reactions described in the literature (14–17). The overall yield of 1 from 1-bromopentane-4,4, 5,5-d_4 was 80%. Deuteriated 2,5-undecadiyn-1-ol (2), the central intermediate, was obtained in 85% yield by reacting the diGrignard complex of propargyl alcohol with 1 in tetrahydrofuran (THF) with cuprous cyanide as catalyst (Scheme 2). The methanesulfonyl ester of compound 2 was synthesized in 97% yield by the procedure described by



SCHEME 1



SCHEME 2

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Abbreviations: GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; THF, tetrahydrofuran.

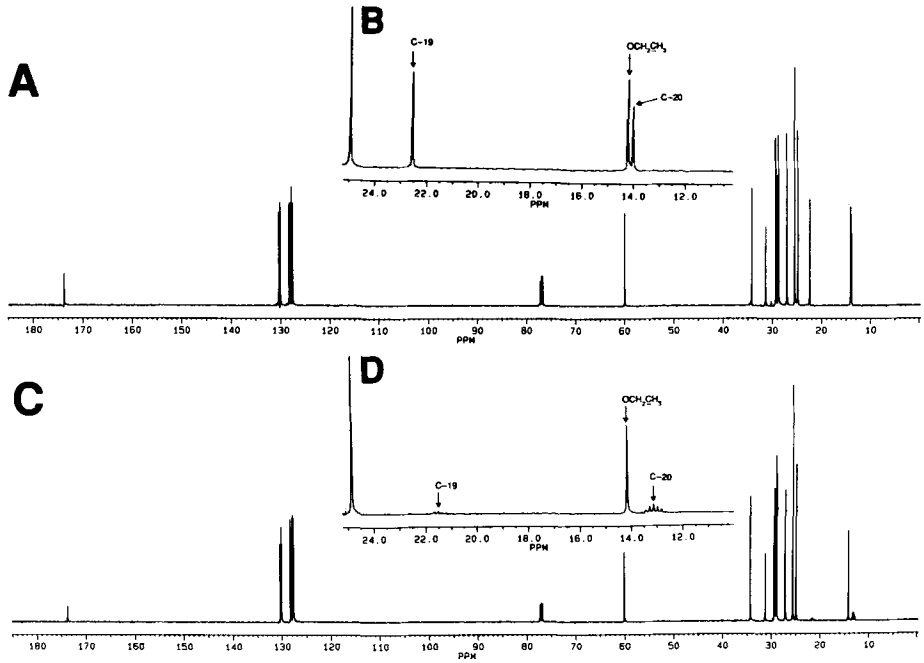

FIG. 1. ^{13}C Nuclear magnetic resonance spectra of ethyl 8,11,14-eicosatrienoate: (A) nondeuterated sample, (B) expansion between 10–25 ppm, (C) deuterated sample, (D) expansion between 10–25 ppm.

Crossland and Servis (18) and coupled directly with the diGrignard adduct of 8-nonynoic acid to give 8,11,14-eicosatriynoic acid-19,19,20,20-d_4 (5). In a similar way, the mesylate of the central intermediate 2 was coupled with 5,8-nonadiynoic acid to give 5,8,11,14-eicosatetraynoic acid-19,19,20,20-d_4 (4). 5,8-Nonadynoic acid was prepared as described by Osbond *et al.* (19), except that the mesylate of propargyl alcohol was used as the coupling agent. In the procedure described by Osbond *et al.* (19), substituted 1-bromo-2-alkynes are coupled with terminal acetylenic acids. Kunau (20) reported that yields could be improved if 1-iodo-2-alkynes were used as coupling agents. In his procedure, substituted 2-alkyn-1-ols were converted to tosylates and then to iodides (20). In our study, we show that mesylates can be directly coupled with alkynoic acids, as 4 and 5 were obtained in yields of 40 and 45%, respectively. Upon reduction with Lindlar's catalyst, these acetylenic acids gave the acids 6 and 7. The purity of these compounds was estimated by gas–liquid chromatography (GLC) after conversion to the ethyl esters 8 and 9 as 89 and 93%, respectively. Final purification was achieved by silver nitrate thin-layer chromatography. Deuteriated ethyl arachidonate and ethyl dihomo-γ-linolenate were characterized by ^1H and ^{13}C nuclear magnetic resonance (NMR) and mass spectral analysis (21–24). The signals for carbons (C-19 and C-20) bearing two deuteriums were diminished significantly, such that they were hardly detected (Figs. 1 and 2). Thus, no peaks for terminal carbons (C-19 and C-20) were observed in the ^{13}C NMR of deuteriated compounds 8 and 9.

EXPERIMENTAL PROCEDURES

Reagents. The following compounds and reagents were used as received: 4-pentynol was purchased from Farchan Laboratories (Gainesville, FL), *tris*(triphenylphosphine)-chlororhodium from Strem Chemicals (Newburyport, MA), methanesulfonyl chloride from Mallinckrodt Inc. (Paris, KY), magnesium from J.T. Baker Inc. (Phillipsburg, NJ), Lindlar catalyst from Fluka Chemical Co. (Ronkonkoma, NY) and lithium aluminum hydride from Aldrich Chemical Company (Milwaukee, WI). THF was freshly distilled over $LiAlH_4$.

Gas chromatography. A 30 m × 0.25 mm DB-WAX capillary column (i.d., 0.25 mm) from J&W Scientific (Folsom, CA) was used to analyze the purity of compounds.

^{13}C and ^1H NMR. NMR spectra were recorded with a Bruker AM 500 pulsed Fourier transform spectrometer (Karlsruhe, Germany) operating at 125.69 MHz for ^{13}C and 500 MHz for ^1H at ambient temperature. For ^{13}C, *ca.* 7500 transients were collected on solutions in $CDCl_3$, which served as both the internal lock and the secondary reference, using 5 mm tubes. Sweep widths of 250 ppm and 32K data points limited acquisition time to 0.49 s and were used to obtain chemical shifts within ± 1.2 Hz, *i.e.*, ± 0.008 ppm. A pulse width of 4 μs (50°) was employed with a delay of 0.3 s between pulses. Decoupling power was held to *ca.* 1W with Waltz 16 composite pulse decoupling, which was used to minimize sample heating. The signals from the carbons bearing two deuterium atoms were diminished (Figs. 1 and 2) to such an extent that they were not observed. Thus no peaks were detected for terminal carbons 19 and 20 for compounds 8 and 9.

Gas chromatography/mass spectrometry. Mass spectrometry was carried out using a Hewlett-Packard (Palo Alto, CA) 5970A mass selective detector equipped with a 30 m × 0.25 mm DB-225 capillary column.

2,5-Undecadiyn-1-ol-10,10,11,11-d_4 (2). Bromoethane (98.1 g, 0.9 mol) in dry THF (75 mL) was added to a stirred

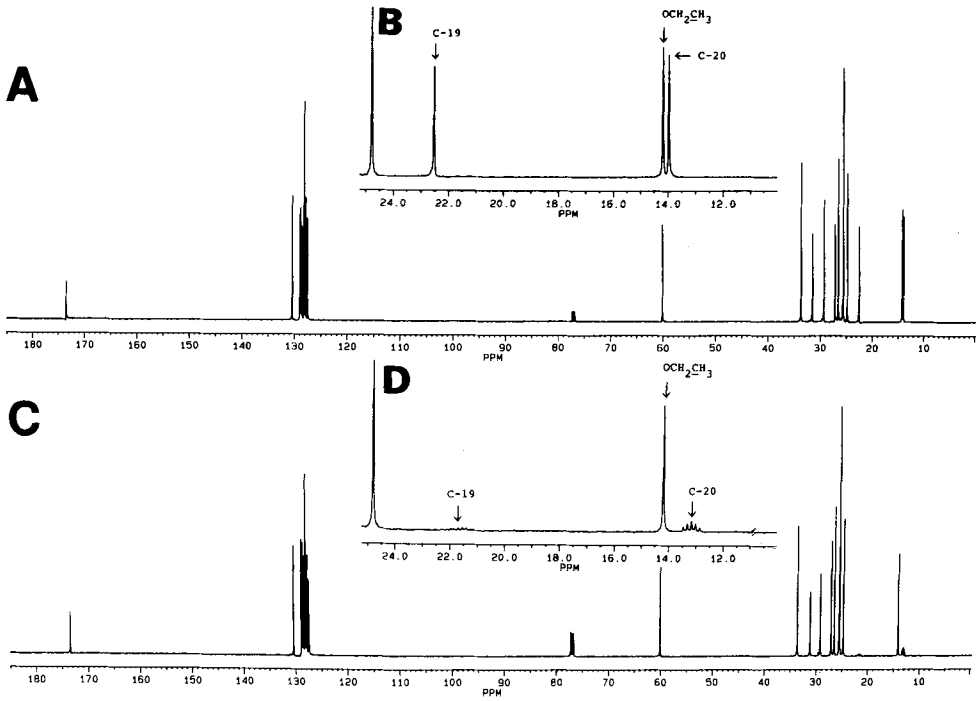

FIG. 2. ^{13}C Nuclear magnetic resonance spectra of ethyl 5,8,11,14-eicosatetraenoate: (A) nondeuterated sample, (B) expansion between 10–25 ppm, (C) deuterated sample, (D) expansion between 10–25 ppm.

suspension of Mg (18.9 g, 0.78 mol) under THF (300 mL) at 0°C. Propargyl alcohol (22.4 g, 0.4 mol) in THF was added to the Grignard reagent with stirring at 3–5°C during 30–45 min, and then at 20°C for 2 h. The solution was cooled to 5°C, cuprous cyanide (200 mg) was added and, after 15 min, 1-bromo-2-octyne-7,7,8,8-d_4 (48 g, 0.25 mol) in THF (75 mL) was added dropwise during 20–30 min. The solution was then allowed to stir at room temperature for *ca.* 3 h and was then heated under gentle reflux overnight (*ca.* 14 h). The reaction mixture was added to 2N H_2SO_4 (*ca.* 600 mL) and ice, extracted with diethyl ether (3 × 200 mL), brine (*ca.* 75 mL), and dried over Na_2SO_4. The alcohol was distilled at 87–89°C at 10^{-3} mm Hg (19) to yield 36 g of 2 (85.7%).

2,5-Undecadiynol-10,10,11,11-d_4 *(3).* Methanesulfonyl chloride (16.1 g, 0.14 mol) was added dropwise to a stirred solution of alcohol 2 (17 g, 0.1 mol) in dichloromethane (350 mL) and triethylamine (18.1 g, 0.18 mol) at 0 to −10°C over a period of 15 min. Stirring for an additional 1.5 h completed the reaction (18). The reaction mixture was transferred to a separatory funnel with the aid of methylene chloride. The mixture was first extracted with water, followed by 10% HCl, saturated bicarbonate solution and brine. Drying of the methylene chloride solution over anhydrous Na_2SO_4, followed by solvent removal under reduced pressure, gave product 3 (24.2 g, 97.2%). This product was used for coupling without further purification.

Ethyl 5,8,11,14-eicosatetraenoate-19,19,20,20-d_4 *(6).* The synthesis of compound 6 was carried out in three steps as described below:

Step 1: 5,8,11,14-Eicosatetraynoic-19,19,20,20-d_4 *acid (4).* Bromoethane (38.1 g, 0.35 mol) in THF (*ca.* 75 mL) was added to a stirred suspension of Mg (6 g, 0.25 mol) in THF (*ca.* 200 mL) at 0°C. 5,8-Nonadiynoic acid in THF (*ca.* 75 mL) was added to the Grignard reagent over a period of 30 min. The mixture was then stirred at 20°C for about 2 h, cooled to 0°C and 200 mg of cuprous cyanide was added. After stirring for 20 min, a solution of 3 in THF (*ca.* 75 mL) was added over 30 min. The mixture was stirred at room temperature for about 3 h and then gently refluxed overnight (*ca.* 14 h). The reaction mixture was cooled and poured into 2N H_2SO_4 (*ca.* 300 mL) and ice, extracted with diethyl ether (3 × 200 mL), washed with 2N HCl (100 mL), water (3 × 100 mL), brine (100 mL) and dried over Na_2SO_4. The product 4 was crystallized with hexane/diethyl ether (90:10, vol/vol), 12 g, 40%.

Step 2: 5,8,11,14-Eicosatetraenoic-19,19,20,20-d_4 *acid (6).* Compound 4 (4 g) in 100 mL ethyl acetate was reduced with hydrogen in the presence of Lindlar's catalyst (1.5 g) and quinoline (1.5 mL) in a hydrogenation apparatus. When hydrogen uptake stopped (100 ± 5% of theroy), the catalyst was removed by vacuum filtration, and the ethyl acetate was distilled under reduced pressure in a rotary evaporator. The product was dissolved in diethyl ether (250 mL), washed with 2N HCl (50 mL), water (2 × 100 mL), brine (50 mL) and dried over Na_2So_4. Removal of solvent yielded product 6 in 97% yield.

Step 3: Ethyl 5,8,11,14-eicosatetraenoate-19,19,20,20-d_4 *(8).* One mL of acetyl chloride was added dropwise at 0–5°C to the stirred solution of compound 6 (1 g) in absolute ethanol (10 mL). The mixture was stirred at ambient temperature overnight (*ca.* 16 h). The mixture was diluted with water (20 mL) and extracted with diethyl ether (2 × 30 mL), washed with brine (15 mL) and dried over anhydrous Na_2SO_4. Removal of solvent under reduced pressure yielded product 8 (1 g, 95%, GLC purity 89%). Compound 8 was purified by silver nitrate thin-layer

chromatography in 65% yield. Deuterium distribution: 0.0% d_0, 0.0% d_1, 1.2% d_2, 4.04% d_3, 94.76% d_4. ^{1}H NMR ($CDCl_3$): δ 5.30-5.41 (*m*, 8H, CH=CH), 4.11 (*q*, 2H, $COOCH_2$), 2.79-2.92 (*m*, 6H, $C{=}CCH_2C{=}C$), 2.29 (*t*, 2H, CH_2COO), 2.08-2.12 (*m*, 2H, $C{=}CCH_2$), 2.06-2.02 (*m*, 2H, $CH_2C{=}C$), 1.66-1.72 (*m*, 2H, CH_2CH_2COO), 1.27-1.38 (*m*, 4H, $\{CH_2\}_2$), 1.24 (*t*, 3H, OCH_2CH_3), 0.82 (*s*, 1H, CD_2H). ^{13}C NMR (ppm): C-1, 173.5; C-2, 33.7; C-3, 24.78; C-4, 26.5; C-5, 128.9; C-6, 128.8; C-7, C-10, C-13, 25.6; C-8, 128.1; C-9, 128.1; C-11, 127.8; C-12, 128.5, C-14, 127.5; C-15, 130.4; C-16, 27.2; C-17, 29.2, C-18, 31.2; OCH_2, 60.0; OCH_2CH_3, 14.2.

Ethyl 8,11,14-eicosatrienoate-19,19,20,20-d_4 *(9).* Compound **9** was prepared by coupling 8-nonynoic acid with compound **3** to give compound **5** in 45% yield. This, upon Lindlar's reduction, furnished compound **7**, which was converted to the ethyl ester **9** by ethanolic HCl. GLC analysis of the ethyl ester indicated its purity as 93%. Purification of 1 g of this product by silver nitrate thin-layer chromatography yielded 700 mg of the product. Deuterium distribution: 0.04% d_0, 0.17% d_1, 4.17% d_2, 3.69% d_3, 91.92% d_4. ^{1}H NMR ($CDCl_3$): δ 5.34-5.37 (*m*, 6H, CH=CH), 4.10 (*q*, 2H, $COOCH_2$), 2.74-2.78 (*m*, 4H, $C{=}CCH_2C{=}C$), 2.26 (*t*, 2H, CH_2COO), 2.03-2.06 (*m*, 4H, $CH_2C{=}C$), 1.59-1.62 (*m*, 2H, CH_2CH_2COO), 1.21-1.34 (*m*, 13H, $\{CH_2\}_5$ and OCH_2CH_3), 0.82 (*s*, 1H, CD_2H). ^{13}C NMR (ppm): C-1, 173.6; C-2, 34.2; C-3, 24.9; C-4, 28.8; C-5, 29.0; C-6, 29.2; C-7, 27.1; C-8, 130.0; C-9, 127.8; C-10, C-13, 25.6; C-11, 128.1; C-12 128.2; C-14, 127.6; C-15, 130.3; C-16, 27.1, C-17, 29.4; C-18, 31.2; OCH_2, 60.0; OCH_2CH_3, 14.2.

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METHOD

Continuous Monitoring of Lipid Peroxidation by Measuring Conjugated Diene Formation in an Aqueous Liposome Suspension

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A method is described for the direct and continuous monitoring of lipid peroxidation in an aqueous suspension of sonicated liposomes. By means of ultraviolet difference spectroscopy using tandem cuvettes, the formation of conjugated dienes during liposome peroxidation can be followed. Using this technique, the effect of the fatty acid composition of liposomes on lipid peroxidation can be studied. The results show that both the extent and the time scale of lipid peroxidation are influenced by the fatty acid composition of the phosholipid liposomes. This was confirmed also by other methods, such as measurement of the formation of lipid hydroperoxides and measurement of the decrease in polyunsaturated fatty acids. The advantage of the method described is the direct and continuous monitoring of phospholipid peroxidation in an aqueous environment, without subsampling and extraction of peroxidation products into organic solvents. Using this experimental approach based on difference spectra the contributions from changes in liposome, $CuSO_4$ and H_2O_2 concentrations are canceled, thus improving sensitivity. The method can be employed for measuring the susceptibility to peroxidation of membrane phospholipids from fatty acid modified endothelial cells.
Lipids 28, 857–861 (1993).

Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids by free radical reactions (1). The polyunsaturated fatty acids in biological membranes are very susceptible to lipid peroxidation, and their oxidation is thought to be involved in various physiological and pathological phenomena, such as the formation of local messengers (prostaglandins) during tissue activation, cellular aging, radiation damage, mutagenesis, carcinogenesis and atherogenesis (1–4). Radical chain reactions involving polyunsaturated fatty acids cause an initial rearrangement of the double bonds leading to conjugated dienes. Upon reaction with oxygen, lipid hydroperoxides are formed, which readily decompose into various other products including malondialdehyde.

Several methods for measuring lipid peroxidation have been developed (1,3). Lipid peroxidation is usually assessed by measuring the major initial peroxidation products (conjugated dienes, lipid hydroperoxides) and/or minor breakdown products (malondialdehyde, hexanal, volatile hydrocarbons) (3). As proteins and DNA can interfere with the accurate determination of lipid peroxidation, lipids are usually extracted from tissues or cells prior to measurement of peroxidation.

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Abbreviations: BHT, butylated hydroxytoluene; TBARS, thiobarbituric acid reactive substances; PC, phosphatidylcholine; PL, phospholipid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

The methods commonly used for determining conjugated dienes are based on measuring their absorption at 233–236 nm (3,5,6). Sensitivity is limited by the fact that the diene absorption appears as a rather imprecise shoulder on the strong absorption peak of the unperoxidized fatty acid itself. This is especially problematic when small amounts of conjugated dienes have to be determined. By using second derivative spectra, sensitivity can be increased (7). These methods, however, usually involve discontinuous end point analyses in organic solvents, and the lipids need to be extracted first. We therefore developed a method for the continuous monitoring of liposome peroxidation in an aqueous environment, based on the method for measuring low-density lipoprotein peroxidation according to Esterbauer *et al.* (8) and the use of tandem cuvettes to measure the formation of conjugated dienes by ultraviolet (UV) difference spectroscopy. Lipid liposomes were used as a model membrane system to assess lipid peroxidation in aqueous environment (2). Using this technique, we determined the effect of liposome fatty acid composition on lipid peroxidation profiles. The results were compared with those obtained by other methods for monitoring lipid peroxidation, such as the formation of lipid hydroperoxides and the decrease in polyunsaturated fatty acids.

MATERIALS AND METHODS

1-Palmitoyl-2-palmitoleoyl-*sn*-glycero-3-phosphocholine (16:0/16:1 PC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (18:0/18:1 PC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0/18:2 PC), 1-stearyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (18:0/20:4 PC), and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO). 1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2/18:2 PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (18:3/18:3 PC) and 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (20:4/20:4 PC) were from Applied Science Laboratories (Deerfield, IL). Hydrogen peroxide (30% solution) was from Baker B.V. (Deventer, The Netherlands). $GuSO_4$ was from Merck (Darmstadt, Germany). All other reagents were of the highest grade of purity available.

The susceptibility for peroxidation of different lipid samples was determined by measuring conjugated diene formation in aqueous environment according to an adaptation of the method described by Esterbauer *et al.* (8) for low-density lipoproteins. In our setup, we introduced sonicated phospholipid (PL) liposomes and tandem cuvettes for UV difference spectroscopy. The reasoning behind the choice for tandem cuvettes is that we were interested in measuring the optical signal obtained upon interaction between lipids and $CuSO_4/H_2O_2$, which are both UV absorbing. Tandem cuvettes allowed for comparison of the

same amounts of lipid and $CuSO_4/H_2O_2$ mixed in the sample beam and separated in the reference beam.

Lipid liposomes were peroxidized by 200 μM $CuSO_4$ and 2 mM H_2O_2, as described by Kuypers *et al.*(9), and the UV spectrum was monitored continuously by difference spectroscopy using tandem cuvettes (2 cm, 232QS; Hellma B.V., The Hague, The Netherlands). Absorption spectra were recorded on a Uvikon 860 spectrophotometer equipped for data handling (Kontron Instruments, Zürich, Switzerland). UV difference spectra (205–300 nm) were recorded every 5 min for the time periods indicated. By using the curve overlay option, curves were plotted in one figure.

For the preparation of the sonicated liposomes, PLs (300–500 nmol) in methanol were dried in a glass tube by a stream of nitrogen. In case of 18:2/18:2 PC, 18:3/18:3 PC and 20:4/20:4 PC, each of these lipids was mixed 1:1 (mol/mol) with 16:0/16:1 PC in order to obtain 500 nmol phosphatidylcholine (PC) giving 500 nmol oxidizable polyunsaturated fatty acid. One mL of 50 μM Tris buffer [50 mM tris(hydroxymethyl)amino methane in water set to pH 7.5 with hydrochloric acid] presaturated with nitrogen was added to the dried lipid and a dispersion was made by vortexing under nitrogen. PL liposomes were prepared by sonication for 10 min under nitrogen while cooling with ice.

Prior to the peroxidation experiments, lipid liposomes were diluted 1:5 (vol/vol) in 50 μM Tris buffer, and 2 mL was added to the first compartment of the tandem cuvettes in both the reference and the sample beam as shown in Figure 1. The second compartments of both tandem cuvettes were filled with 2 mL of 50 μM Tris buffer alone. The temperature of the cuvettes was kept at 37°C. The UV difference spectra (205–300 nm) recorded at this point showed a straight line. Lipid peroxidation was initiated by addition of 20 μL 200 mM H_2O_2 and 20 μL 20 mM $CuSO_4$ to the lipid liposomes in the first compartment of the sample cuvette (final concentrations 2 mM and 200 μM, respectively). To correct for the effects of lipid dilution and of adding H_2O_2 and $CuSO_4$, the same amounts of H_2O_2 and $CuSO_4$ were added to the buffer containing compartment of the reference cuvette, while 40 μL of distilled water was added to both the lipid-containing compartment of the reference cuvette and the buffer-containing compartment of the sample cuvette. UV difference spectra from 205–300 nm were recorded continuously. The final conditions were 60–100 μM PL liposomes, 2 mM H_2O_2 and 200 μM $CuSO_4$. In this setup the formation of conjugated dienes (233–235 nm) from PLs in the sample cuvette was measured against unperoxidized PLs in the reference cuvette. By this experimental design the contributions from changes in the liposome, $CuSO_4$ and H_2O_2 concentrations to the difference spectra was canceled. The method allows for the sensitive and continuous monitoring of conjugated dienes in an aqueous environment without the necessity of subsampling and extraction of the peroxidation products formed.

Lipid hydroperoxides were determined by the iodometric assay described by Hicks and Gebicki (10). The decrease in polyunsaturated fatty acids due to lipid peroxidation was determined by gas–liquid chromatography as described earlier (11). Lipids were extracted from endothelial cell membranes by the method of Bligh and Dyer (12) using BHT as antioxidant, and PLs were separated from neutral lipids by thin-layer chromatography as described before (11), but BHT in the organic solvents was omitted. Phosphorus content of PLs was quantified according to Böttcher *et al.* (13).

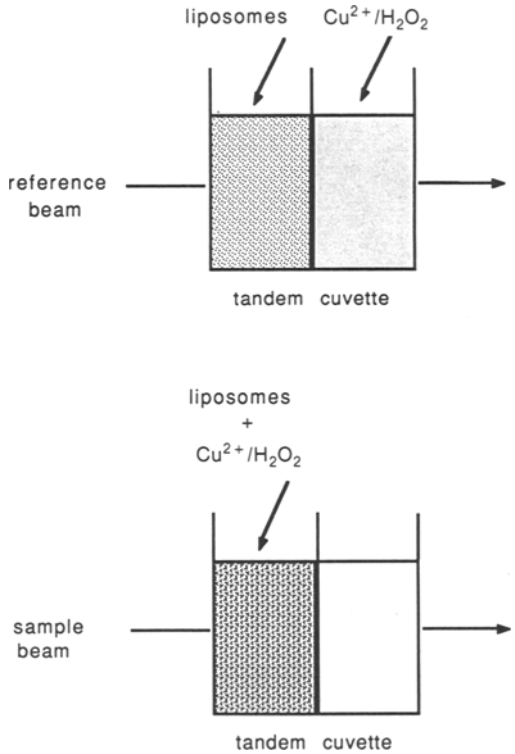

FIG. 1. Ultraviolet difference spectroscopy using tandem cuvettes for monitoring peroxidation of phospholipid liposomes. Peroxidation was started in the sample cuvette by addition of $CuSO_4$ and H_2O_2 to the liposomes. In the reference cuvette, no liposome peroxidation was initiated as $CuSO_4$ and H_2O_2 were added to a separate compartment. The difference spectra were recorded continuously.

RESULTS AND DISCUSSION

Peroxidation profiles of PC liposomes with different fatty acid compositions. We studied the influence of fatty acid composition on the peroxidation profiles of PC liposomes by UV difference spectroscopy using tandem cuvettes. The peroxidation profiles of 16:0/18:2 PC, 16:0/18:3 PC and 18:0/20:4 PC are shown in Figure 2. Peroxidation of 16:0/18:2 PC resulted in an absorption maximum at 234 nm, which first increased with time and then gradually decreased, representing the formation and decomposition of conjugated dienes. The absorption maximum at 234 nm of 16:0/18:3 PC shifted with time toward 225 mm with the concomitant appearance of a dip at 210 nm. This shift may be explained by formation of alkenals and hydroxyalkenals, typical products of lipid peroxidation with absorption maxima at 220–225 nm (6). Two other absorption maxima at 270 and 280 nm were observed, which probably represent the formation of conjugated trienes (6). Conjugated triene formation started later and was lower than conjugated diene formation. The species 18:0/20:4 PC showed a comparable peroxidation profile, but on a slower time scale and without distinct maxima at 270 and 280 nm, probably because a mixture of conjugated trienes is formed. Conjugated triene formation from 18:0/20:4 PC was much lower than from 16:0/18:3 PC. Surprisingly,

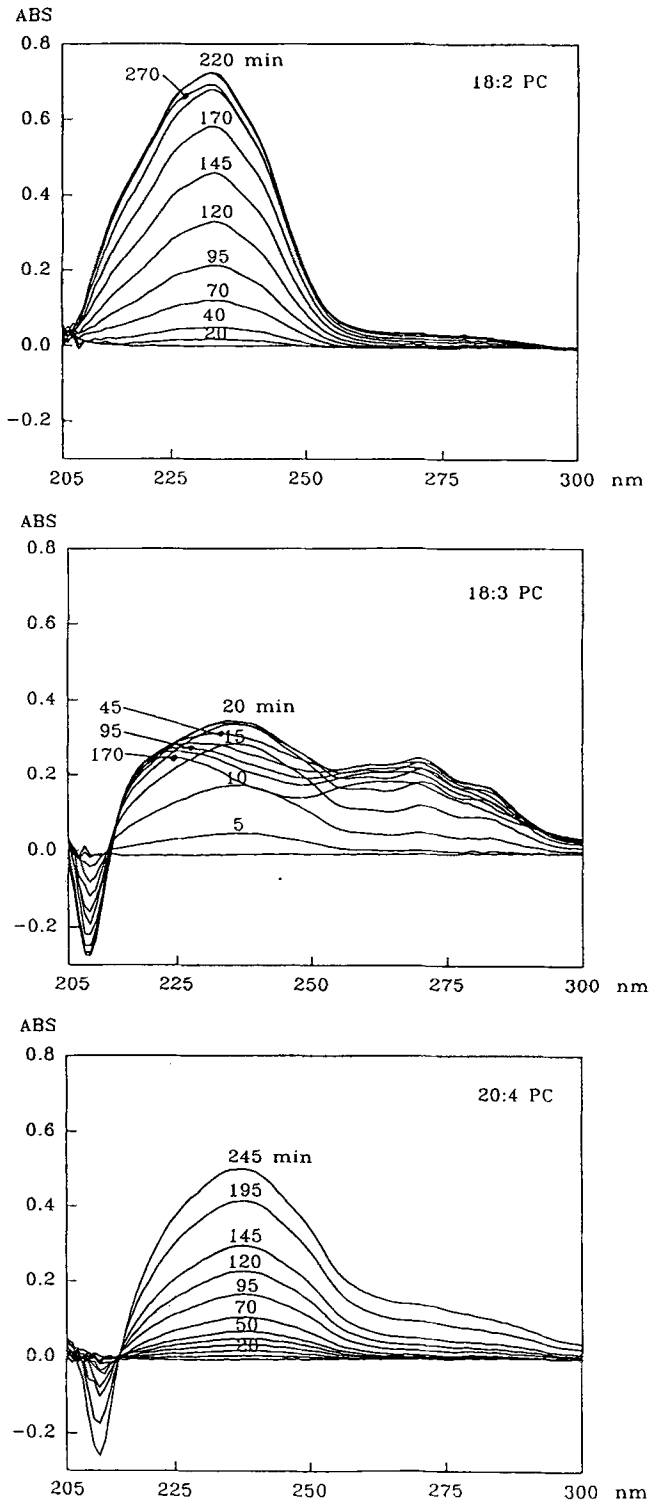

FIG. 2. Change of the ultraviolet (UV) difference spectrum of phosphatidylcholine (PC) liposomes with different fatty acid compositions during peroxidation. Liposomes (100 μM) prepared from 16:0/18:2 PC (top box), 16:0/18:3 PC (middle box) and 18:0/20:4 PC (bottom box) were peroxidized by $CuSO_4$ (200 μM) and H_2O_2 (2 mM). Conjugated diene formation was monitored continuously by UV difference spectroscopy (205–300 nm) using tandem cuvettes. Peroxidation profiles are shown as UV absorption units (ABS) against wavelength (nm) for the time points indicated.

after 250 min of peroxidation, still no decrease in conjugated diene formation was seen with 18:0/4 PC.

The fatty acid composition of the liposomes thus considerably influenced the peroxidation profile (Fig. 2) as well as the time scale of the peroxidation process and the maximal extent of conjugated diene formation (Fig. 3). The 16:0/18:3 PC showed a rapid but relatively low conjugated diene formation, while conjugated diene formation form 16:0/18:2 PC was slower but relatively high in amount. The 18:0/20:4 PC showed a slow but progressive formation of conjugated dienes. Both the peroxidation profiles and the maximal extent of conjugated diene formation were reproducible, standard deviations being below 6%. When conjugated diene formation was measured after relatively short peroxidation times, more variable results were observed, possibly because of the presence of small amounts of preexisting lipid peroxides in the samples used.

We also studied the influence of liposome concentration on the lipid peroxidation process (Fig. 4). It can be seen in Figure 4A that there is a linear relationship between the maximal amount of conjugated dienes formed and the amount of 16:0/18:2 PC liposomes present during peroxidation. The extent of lipid peroxidation was dependent on the amounts of $CuSO_4/H_2O_2$ added; addition of $CuSO_4$ or H_2O_2 alone resulted in a much lower peroxidation rate (data not shown). Addition of the antioxidant BHT to the liposomes during preparation resulted in a lag phase at low BHT concentrations, while total inhibition of lipid peroxidation occurred with higher amounts of BHT (Fig. 4B). Similar effects of liposome, $CuSO_4/H_2O_2$ and BHT concentrations were found with 16:0/18:3 PC and 18:0/20:4 PC. We also measured the UV absorption spectra of known amounts of a standard fatty acid-derived conjugated diene (13-hydroxy-9,11-octadecaenoic acid), and

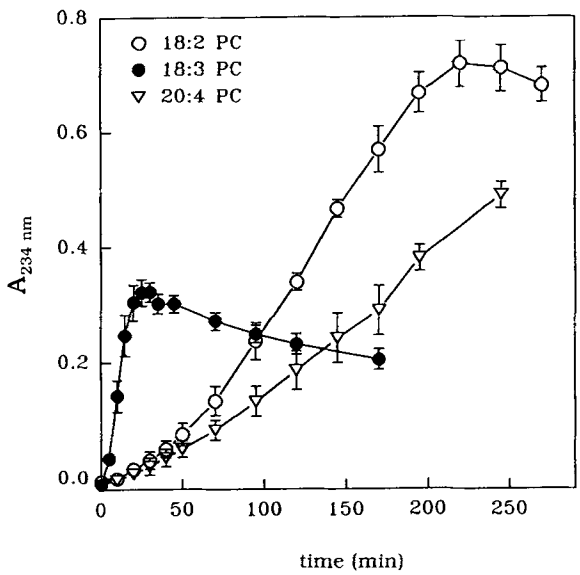

FIG. 3. Time scale of conjugated diene formation of phosphatidylcholine (PC) liposomes with different fatty acid compositions during peroxidation. For procedure details, see Figure 2. Ultraviolet absorption at 234 nm (conjugated diene formation) is plotted against peroxidation time. The mean values ± SD of five separate liposome preparations measured on three different days are shown. Wavelength, nm.

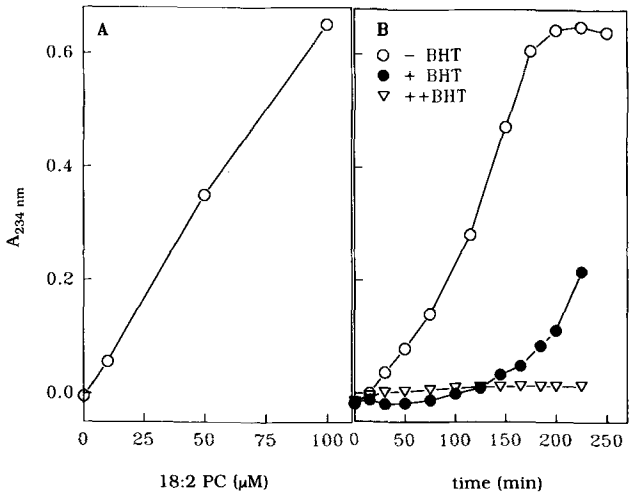

FIG. 4. Influence of liposome concentration and butylated hydroxytoluene (BHT) on conjugated diene formation (for procedural details, see Fig. 2). Figure 4A shows the maximal conjugated diene formation during peroxidation of 16:0/18:2 PC liposomes with a final concentration of 10 μM, 50 μM or 100 μM. In Figure 4B, the influence of BHT [0.05 nmol BHT/nmol 16:0/18:2 phosphatidylcholine (PC) for closed circles and 0.5 nmol BHT/nmol 16:0/18:2 PC for open triangles] on the peroxidation of 100 μM 16:0/18:2 PC is shown. Wavelength, nm.

found a linear increase in absorption at 234 nm with increasing amounts (data not shown).

As expected, no formation of conjugated dienes was observed with liposomes consisting of 16:0/16:1 PC or 18:0/18:1 PC. When we recorded peroxidation profiles of 18:2/18:2 PC 1:1 mixed with 16:0/18:1 PC, no differences were found with that of 16:0/18:2 PC. In both liposome preparations, the amount of oxidizable linoleic acid is equal. These observations suggest that only the polyunsaturated fatty acids are peroxidized within the time scale used, and that the amount, but not the position, of the polyunsaturated fatty acid within the PL molecule influences lipid peroxidation in these liposomes.

Comparison of the continuous conjugated diene measurement with other discontinuous methods for estimating lipid peroxidation in vitro. We compared our conjugated diene measurement with other lipid peroxidation assays, such as measurement of the decrease in polyunsaturated fatty acids by gas-liquid chromatography and measurement of the formation of lipid hydroperoxides by an iodometric assay.

The formation of conjugated dienes (as a measure for initial lipid peroxidation) and the decrease in polyunsaturated fatty acids (as a measure of total lipid peroxidation) for liposomes containing 16:0/18:2 PC, 16:0/18:3 PC or 18:0/20:4 PC was measured after 80 min of peroxidation. The relation between both methods is shown in Table 1. We also measured the sensitivity to peroxidation of PLs obtained from fatty acid modified endothelial cells. As a lipid source, we used PLs which were extracted from endothelial cell membranes having different fatty acid compositions. Fatty acid modified endothelial cells were cultured as described before (11). Liposomes (60 μM phospholipid) were prepared from endothelial phospholipids with (A) a normal fatty acid composition, (B) an increased oleic acid content, (C) an increased linoleic acid and arachidonic acid content or (D) an increased eicosapentaenoic acid and docosahexaenoic acid content. (The fatty acid compositions are shown in Table 2). The liposomes were peroxidized for 80 min with 2 mM H_2O_2 and 200 μM $CuSO_4$ (final concentrations), and conjugated diene formation was measured continuously. As an example Figure 5A shows the peroxidation profile of liposomes prepared from endothelial cells with a normal fatty acid composition, and Figure 5B shows that of liposomes obtained from cells with increased oleic acid content. both difference spectra obtained during peroxidation showed characteristics of the 18:0/20:4 PC and 16:0/18:2 PC spectra (Fig. 2). This was to be expected for cellular PLs containing a mixture of various fatty acids, because PC comprises 53.4% of total PLs (11), and linoleic acid and arachidonic acid are the most abundant polyunsaturated fatty acids in these endothelial cells (Table 2). Figure 5 shows that the extent of conjugated diene formation in liposomes with increased oleic acid content was decreased compared to liposomes with normal fatty acid composition. As was shown earlier (11), the polyunsaturated fatty acid content of the membrane PLs was decreased when the oleic acid content increased. Table 2 shows that endothelial cell PLs with normal fatty acid composition (A) contained 0.29 nmol linoleic acid and 0.21 nmol arachidonic acid, while cells modified with oleic acid (B) contained 0.15 nmol linoleic acid and 0.13 nmol arachidonic acid in their membrane PLs. Probably the decreased availability of the polyunsaturated fatty acids linoleic acid and arachidonic acid

TABLE 1

Conjugated Diene Formation and Decrease of Polyunsaturated Fatty Acids During Peroxidation of Phosphatidylcholine (PC) Liposomes with Different Fatty Acid Compositions[a]

Liposomes	Decreased fatty acids nmol/nmol PL	Conjugated dienes A_{234}
15:0/18:2 PC	0.49	0.21
16:0/18:3 PC	0.74	0.26
18:0/20:4 PC	0.23	0.11

[a]Liposomes [100 μM phospholipids (PL)] were prepared and peroxidized for 80 min by addition of $CuSO_4$ (200 μM) and H_2O_2 (2 mM). Conjugated diene formation (absorption at 234 nm) was monitored continuously by ultraviolet difference spectroscopy using tandem cuvettes. Decrease in polyunsaturated fatty acids (nmol/nmol PL) was measured by gas-liquid chromatography.

TABLE 2

Fatty Acid Composition of Phospholipids (PL) Extracted from Fatty Acid-Modified Endothelial Cells[a]

Fatty acids	nmol/nmol PL			
	A	B	C	D
18:1n-9	0.33	0.67	0.20	0.29
18:2n-6	0.29	0.15	0.35	0.24
20:4n-6	0.21	0.13	0.30	0.11
20:5n-3	0.01	—	—	0.12
22:6n-3	0.06	0.04	0.01	0.11

[a]Fatty acid composition (nmol/nmol PL) of endothelial phospholipids with (A) a normal fatty acid composition, (B) an increased oleic acid content, (C) an increased linoleic acid and arachidonic acid content or (D) an increased eicosapentaenoic acid and docosahexaenoic acid content. Cells were cultured as described before (11).

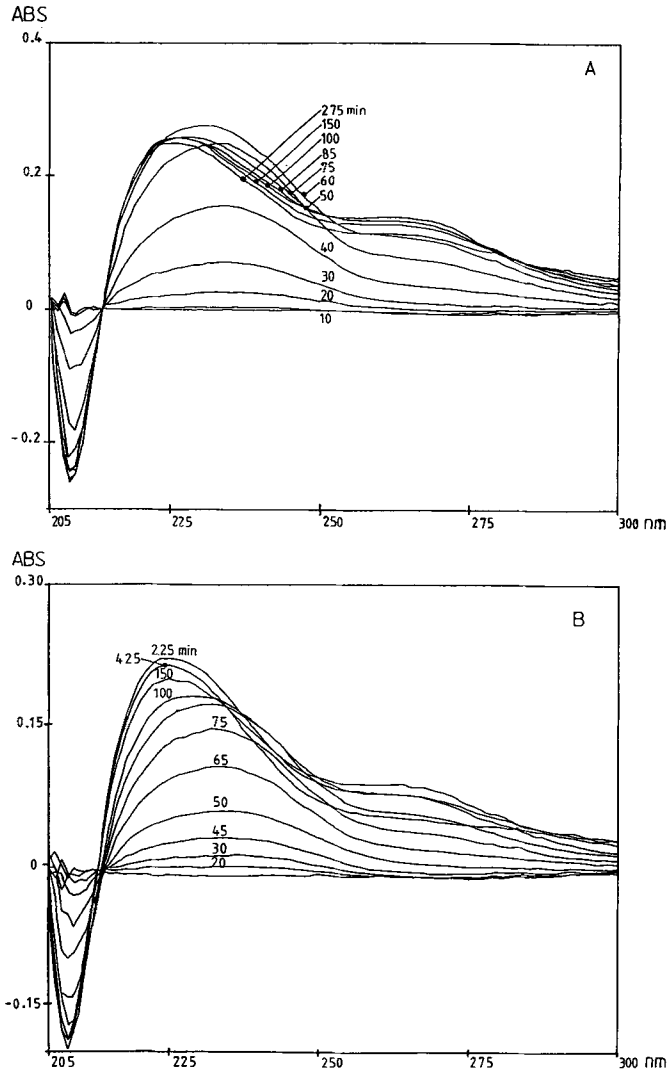

FIG. 5. Change of the ultraviolet (UV) difference spectrum during peroxidation of liposomes prepared from endothelial cell phospholipids. Phospholipids were extracted from endothelial cells with normal fatty acid composition or with increased oleic acid content and liposomes were prepared. Peroxidation with $CuSO_4$ and H_2O_2 was monitored by UV difference spectroscopy as in Figure 2. Wavelength, nm; absorption units, ABS.

was reflected in a decreased formation of conjugated dienes in these liposomes, as oleic acid itself cannot form conjugated dienes.

In the same batch of endothelial cell-derived liposomes, the amount of lipid hydroperoxides formed after 80 min of peroxidation was measured. Also the fatty acid compositions of both peroxidized (80 min) and nonperoxidized liposomes were determined, and the decrease in polyunsaturated fatty acids during peroxidation was calculated. The relation between the measurement of conjugated diene formation, the decrease in polyunsaturated fatty acids and the formation of lipid hydroperoxides during 80 min of peroxidation are shown in Table 3. It is also shown in Table 3 that the fatty acid composition of the endothelial cell PLs influenced the extent of lipid peroxidation as measured by three different methods.

In conclusion, we describe here a method for the continuous monitoring of lipid peroxidation by measuring conjugated diene formation by UV difference spectroscopy using tandem cuvettes and sonicated liposomes. Our method allows the direct and continuous monitoring of PL peroxidation in an aqueous environment, without the disadvantages of subsampling and extraction of peroxidation products into organic solvents. We have shown that the time course and extent of lipid peroxidation are considerably affected by the fatty acid composition of the PL liposomes. This was confirmed by other methods for measuring lipid peroxidation, such as by measuring the decrease in polyunsaturated fatty acids and the formation of lipid hydroperoxides.

TABLE 3

Conjugated Diene Formation, Lipid Hydroperoxide Formation and Decrease in Polyunsaturated Fatty Acids During Peroxidation of Endothelial Cell Phospholipids (PL)[a]

Liposomes	Decreased fatty acids nmol/nmol PL	Lipid hydroperoxides nmol/nmol PL	Conjugated dienes A_{234}
A	0.265	0.0054	0.287
B	0.161	0.0036	0.173
C	0.323	0.0108	0.315
D	0.282	0.0064	0.285

[a]PLs were extracted from endothelial cells with different fatty acid compositions: (A) cells with normal fatty acid composition, (B) cells with increased oleic acid (n-9) content, (C) cells with increased linoleic and arachidonic acid (n-6) content and (D) cells with increased eicosapentaenoic and docosahexaenoic acid (n-3) content. Liposomes (60 μM PL) were prepared and peroxidized for 80 min. Conjugated dienes, lipid hydroperoxides and decrease in polyunsaturated fatty acids were measured.

ACKNOWLEDGMENTS

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Separation of Bile Acid Methyl Esters by High-Performance Liquid Chromatography

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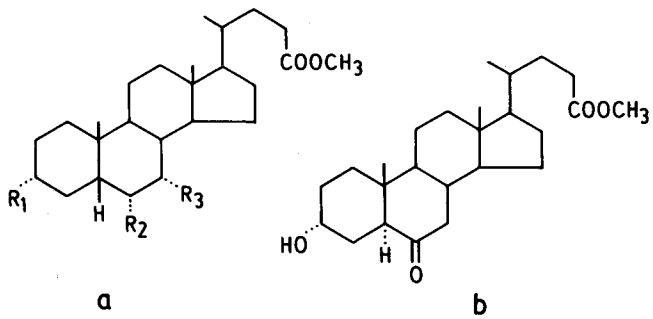
Conjugated bile acids, namely glyco- and tauro-3α,6α-dihydroxy-5β-cholanoic acid (hyodeoxycholic acid), 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid), 3α,6α,7α-trihydroxy-5β-cholanoic acid (hyocholic acid) and 3α-hydroxy-6-oxo-5β-cholanoic acid (6-keto-litocholic acid) were isolated from pig bile, and subsequently transformed into the corresponding methyl esters. Separation of the methyl esters of the isolated bile acids by high-performance liquid chromatography (HPLC) was accomplished on a ZORBAX-CN column (Dupont, Boston, MA) with *n*-hexane/2-propanol/methylene chloride (89:6:5, by vol) as the mobile phase containing traces (≈1%) of amyl alcohol and water as moderators. HPLC analysis of the methyl esters also showed the presence of methyl 3α-hydroxy-6-oxo-5α-cholanoate, which was probably produced in the course of alkaline hydrolysis of the conjugated bile acids. ***Lipids 28,*** **863–865 (1993).**

3α,6α-Dihydroxy-5β-cholanoic acid, which makes up about 50% of pig bile, is often used as starting material for the synthesis of progesterone (1–3). Isolation of 3α,6α-dihydroxy-5β-chlanoic acid from pig bile is based on the crystallization of the adduct between methyl 3α,6α-dihydroxy-5β-cholanoate and benzene (4). To monitor the course of isolation of methyl 3α,6α-dihydroxy-5β-cholanoate, a procedure was developed for the separation of the mixtures of methyl esters of bile acids present in pig bile.

There have been a number of papers (5–7) dealing with the separation and analysis of methyl esters of bile acids by high-performance liquid chromatography (HPLC). Jefferson and Chang (5) and Iida *et al.* (6) described in detail both qualitative and quantitative analyses of some synthetic methyl esters of mono-, di- and trihydroxy-cholanoic acids, as well as of keto-cholanoic acids. Tint *et al.* (7) reported a method for the separation methyl esters of the bile acids from the *Alligator mississippiensis.* Scheme 1 shows the structures of the various bile acid methyl esters obtained from pig bile.



a

$R_1 = OH$ $R_2 = OH$ $R_3 = H$
methyl 3α,6α-dihydroxy-5β-cholanoate

$R_1 = OH$ $R_2 = H$ $R_3 = OH$
methyl 3α,7α-dihydroxy-5β-cholanoate

$R_1 = OH$ $R_2 = OH$ $R_3 = OH$
methyl 3α,6α,7α-trihydroxy-5β-cholanoate

$R_1 = OH$ $R_2 = O$ $R_3 = H$
methyl 3α-hydroxy-6-oxo-5β-cholanoate

b

methyl 3α-hydroxy-6-oxo-5α-cholanoate

SCHEME 1

MATERIALS AND METHODS

Equipment. Methyl esters of pig bile acids were separated on a Dupont (Boston, MA) ZORBAX-CN column (25 cm × 4.6 mm i.d.) using a Waters Associates (Milford, MA) HPLC Model 6000 A, and a Waters Model IR 401 differential refractometer for detection (sensitivity: ×8), and a Kipp & Zonen (Delft, Holland) recorder (1 and 2 mm/min).

Synthesis of standard methyl esters of bile acids. Methyl 3α,6α-dihydroxy-5β-cholanoate, methyl 3α,7α-dihydroxy-5β-cholanoate, 3α,6α,7α-trihydroxy-5β-cholanoate, 3α-hydroxy-6-oxo-5β-cholanoate and 3α-hydroxy-6-oxo-5α-cholanoate were prepared from free bile acids, purchased from Sigma Chemical Co. (St. Louis, MO), by treatment with diazomethane.

Isolation of a mixture of conjugated bile acids from pig bile and their conversion to methyl esters. Sodium salts of the conjugated bile acids were precipitated from fresh pig bile with saturated aqueous sodium chloride (8). Conjugated bile acids (200 g) were hydrolyzed with KOH (100 g) in a mixture of ethyleneglycol (1000 mL) and water (500 mL) at 140°C for 10 h. After acidification with HCl (1:1) to pH 1, an oily product was obtained which, after solidification, was separated by filtration, air-dried and finally ground with mortar and pestle. The product (130 g) thus obtained was a mixture of free bile acids.

Free bile acids (90 g) were esterified with methanol (700 mL) in the presence of perchloric acid (10 mL) at room temperature for 12 h. The course of esterification was followed by thin-layer chromatography using benzene/isopropanol (85:15, vol/vol) as developing solvent. After the reaction was completed, the solution was neutralized, diluted with water and the methyl esters were extracted with diethyl ether. Yield of the mixture of crude methyl esters was 90 g.

Preparation of methyl 3α,6α-dihydroxy-5β-cholanoate. A mixture of crude methyl esters of pig bile acids (90 g) was refluxed in benzene (300 mL) for 1 h. The clear reaction mixture was left at room temperature, and after 3 d methyl 3α,6α-dihydroxy-5β-cholanoate was crystallized as a benzene adduct. The crystals were separated by filtration, and the filtrate was evaporated to dryness. The oily residue was again dissolved in benzene, and methyl 3α,6α-

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Abbreviation: HPLC, high-performance liquid chromatography.

dihydroxy-5β-cholanoate was crystallized. After removal of the crystals, the procedure was repeated. Pure methyl 3α,6α-dihydroxy-5β-cholanoate was obtained in a yield of 32.6% (calculated based on the mixture of crude methyl esters of pig bile acids); m.p. 114°C. *All procedures involving benzene were carried out in a well-ventilated hood.*

RESULTS AND DISCUSSION

A procedure for the separation of the methyl esters of crude pig bile acids has been developed to follow the course of the isolation of methyl 3α,6α-dihydroxy-5β-cholanoate from this mixture.

To optimize conditions for HPLC analysis, experiments were first carried out with mixtures of standard synthetic methyl esters, which included methyl 3α-hydroxy-6-oxo-5α-cholanoate, methyl 3α,7α-dihydroxy-5β-cholanoate, methyl 3α-hydroxy-6-oxo-5β-cholanoate, methyl 3α,6α-dihydroxy-5β-cholanoate and methyl 3α,6α,7α-trihydroxy-5β-cholanoate. Figure 1 shows the chromatogram of this mixture on a ZORBAX-CN column, using *n*-hexane/2-propanol/methylene chloride (89:6:5, by vol) as mobile phase with traces of water and amyl alcohol (≈1%) as moderators. Under these conditions, an efficient separation of these methyl esters was achieved in less than 30 min.

Methyl 3α-hydroxy-6-oxo-5α-cholanoate had a retention time of 7.52 min, followed by methyl 3α,7α-dihydroxy-5β-cholanoate (9.17 min) and methyl 3α-hydroxy-6-oxo-5β-cholanoate (12.16 min). Further elution at an isobaric flow rate of 1.5 mL/min resulted in the appearance of methyl 3α,6α-dihydroxy-5β-cholanoate and methyl 3α,6α,7α-trihydroxy-5β-cholanoate at the retention times of 16.59 and 21.30 min, respectively.

The elution profile of a mixture of crude methyl esters of pig bile acids is presented in Figure 2 (left panel). Figure 2 (center and right panels) shows the elution profiles of the filtrates from the first and second recrystallization of methyl 3α,6α-dihydroxy-5β-cholanoate. The relative

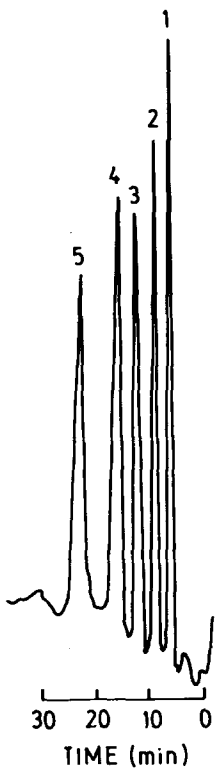

FIG. 1. Elution profile of a standard solution of bile acid methyl esters on a ZORBAX-CN column with *n*-hexane/2-propanol/methylene chloride (89:6:5, by vol) as mobile phase with traces (≈1%) of amyl alcohol and water as moderators. Isobaric flow rate, 1.5 mL/min; detection, differential refractometry. Peaks: 1, methyl 3α-hydroxy-6-oxo-5α-cholanoate; 2, methyl 3α,7α-dihydroxy-5β-cholanoate; 3, methyl 3α-hydroxy-6-oxo-5β-cholanoate; 4, methyl 3α,6α-dihydroxy-5β-cholanoate; 5, methyl 3α,6α,7α-trihydroxy-5β-cholanoate.

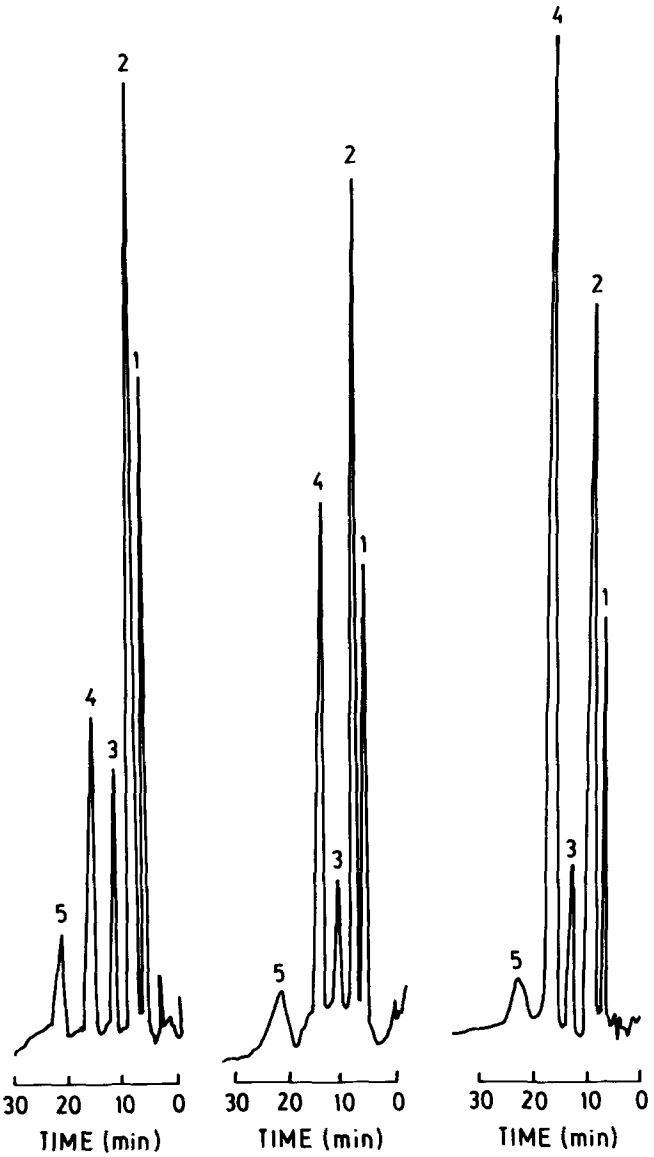

FIG. 2. High-performance liquid chromatography separation of the mixture of methyl esters obtained by esterification of bile acids isolated from pig bile (left panel); bile acids from filtrate after first crystallization of methyl 3α,6α-dihydroxy-5β-cholanoate (center panel); bile acids from filtrate after second crystallization of methyl 3α,6α-dihydroxy-5β-cholanoate (right panel). Peak designations and chromatographic conditions are as in Figure 1.

TABLE 1

Relative Percentage of Methyl Esters of Bile Acids in Pig Bile Fractions

Methyl ester	Crude mixture	Filtrate after first crystallization	Filtrate after second crystallization
Methyl 3α-hydroxy-6-oxo-5α-cholanoate	10.53	15.76	17.95
Methyl 3α,7α-dihydroxy-5β-cholanoate	26.85	32.23	43.21
Methyl 3α-hydroxy-6-oxo-5β-cholanoate	6.54	8.94	10.46
Methyl 3α,6α-dihydroxy-5β-cholanoate	48.25	28.23	19.07
Methyl 3α,6α,7a-trihydroxy-5β-cholanoate	5.82	8.82	9.30

percentages of individual methyl esters of bile acids in the mixture of methyl esters of crude pig bile acids and in the filtrates from first and second recrystallization are given in Table 1.

Application of HPLC under the conditions described resulted in a good separation of methyl 3α-hydroxy-6-oxo-5α-cholanoate, methyl 3α,7α-dihydroxy-5β-cholanoate, methyl 3α-hydroxy-6-oxo-5β-cholanoate, methyl 3α,6α-dihydroxy-5β-cholanoate and methyl 3α,6α,7α-trihydroxy-5β-cholanoate. Because of its accuracy and simplicity, the method has been proven useful in monitoring the preparative isolation of 3α,6α-dihydroxy-5β-cholanoic acid from pig bile.

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ARTICLE

Modulation by Sphingosine of Substrate Phosphorylation by Protein Kinase C in Bovine Mammary Gland

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The effect of sphingosine on the phosphorylation of endogenous proteins by protein kinase C (PKC) was investigated in bovine mammary gland. Several proteins were shown to be substrates for PKC in both cytosolic and total particulate fractions by phosphorylation in the absence or presence of 1-oleoyl-2-acetyl-*sn*-glycerol, phosphatidylserine (PS) and Ca^{2+}. At concentrations of 83 μM or less, sphingosine inhibited phosphorylation of several substrates for PKC in both fractions. Phosphorylation of cytosolic 36 kDa, 21 kDa and particulate 36 kDa proteins was particularly sensitive to sphingosine. Cytosolic 97 kDa phosphorylation (which was enhanced by Ca^{2+} alone) was also sensitive to sphingosine. The inhibition was reversed by excess addition of lipid cofactors, particularly PS, but not by Ca^{2+}. At higher concentrations (167 and 417 μM), in addition to the inhibition seen at lower concentrations, sphingosine stimulated phosphorylation of several proteins, including cytosolic 19 kDa and particulate 53 kDa, which were not detected in the absence of sphingosine. The sphingosine-induced phosphorylation disappeared with excess addition of PS, but not with addition of Ca^{2+}. The results point toward the importance of the interaction of sphingosine with membrane phospholipids in the signal transduction pathway mediated by PKC-dependent phosphorylation in bovine mammary gland.
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Mammary gland is a tissue rich in glycosphingolipids, and bovine milk is known to be rich in the gangliosides GD3 and GM3, as constituents of the milk fat globule membrane derived from the plasma membrane of secretory epithelial cells (1,2). Glycosphingolipids are located at the cell surface and are believed to be important as recognition sites for mediating extracellular signals to intracellular second messengers such as diacylglycerols.

Protein kinase C (PKC) is a key enzyme in the signal transduction pathway, which is activated by the combined action of diacylglycerols, phospholipids and Ca^{2+} (3). Mammary gland PKC is suggested to be important in the gland's function. For example, mouse mammary gland PKC activity is regulated by steroid hormones (4) and is depressed during pregnancy and lactation (5). It also has been reported that mouse mammary gland PKC is translocated by prolactin from cytosol to the particulate fraction (6). Sphingosine, a constituent of glycosphingolipids, inhibits PKC (7), and the inhibition is reversed by phospholipids and diacylglycerols (7,8). The increase in sphingosine concentrations in canine kidney cells induced by the addition of a glucosylceramide synthase inhibitor resulted in reduced PKC activity, as well as in inhibition of cell growth and DNA synthesis (9), suggesting an intimate relationship between endogenous sphingosine concentrations and PKC activity. Phenotypic changes in 3T3 cells are also suggested to be associated with accumulation of sphingosine induced by a ceramide analogue (10).

PKC is thought to function by phosphorylating its intracellular substrate proteins after activation by diacylglycerols which are transiently produced by extracellular signals and translocation from the cytosol to particulate fractions. In bovine mammary gland, several proteins have been identified as substrates for PKC in both cytosolic and total particulate fractions (11). The purpose of the present study was to define the effect of sphingosine on PKC-dependent endogenous protein phosphorylation in bovine mammary gland.

MATERIALS AND METHODS

Materials. D-sphingosine from bovine brain cerebrosides, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), phosphatidylserine (PS), histone H1 (type III-S) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) was from Boehringer (Mannheim, Germany). [γ-^{32}P]adenosine triphosphate (ATP) was from New England Nuclear-DuPont (Wilmington, DE). Cow mammary glands in the lactating state were obtained from a local slaughterhouse. The *pars glandularis* was cut into small blocks and frozen at −80°C until use.

Preparation of subcellular fractions. The tissue was thawed, cut into small pieces and homogenized in 9 vol of 0.25 M sucrose containing 20 mM *tris*(hydroxymethyl)-aminomethane (Tris)-HCl, pH 7.5, 2.5 mM ethyleneglycol *bis*(2-aminoethylether)tetraacetic acid (EGTA), 10 mM 2-mercaptoethanol, 0.5 mM PMSF and 10 μg/mL leupeptin, using a Polytron (Kinematica, Littau, Switzerland). The homogenate was centrifuged at 38,000 × *g* for 60 min; the resultant supernatant is being referred to as cytosol. The pellet was treated with 0.3% Triton X-100 for 60 min on ice, then centrifuged at 38,000 × *g* for 60 min, and the supernatant obtained was used as (solubilized) total particulate fraction.

PKC assay. The standard assay mixture (0.2 mL) contained 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 40 μg of histone, 50 μM [γ-^{32}P]ATP and appropriate amounts of the subcellular proteins, in the absence or presence of OAG (1 μg), PS (5 μg) and Ca^{2+} (1 μM). Ca^{2+}–EGTA buffer was prepared in the presence of 250 μM EGTA (from the enzyme solution) (12). The cofactor concentrations were similar to those used by other investigators (13,14). In particular, the 1 μM Ca^{2+} was physiological, because the intracellular Ca^{2+} concentration was in the micromolar range. Sphingosine suspended in 20 mM Tris-HCl, pH 7.5, was sonicated and added to the mixture at different concentrations as indicated in the figures and tables. The reaction was initiated by the addition of ATP and continued

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Abbreviations: ATP, adenosine triphosphate; DEAE, diethylaminoethyl; EGTA, ethyleneglycol *bis*(2-aminoethylether)tetraacetic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; Tris, *tris*(hydroxymethyl)aminomethane.

for 5 min at 30°C. PKC activity was determined by subtracting basal activity in the absence of cofactors.

Endogenous protein phosphorylation. The reaction conditions for phosphorylation were the same as those described for the PKC assay except for the deletion of histone from the mixture. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done using a 12% gel. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried and exposed to Kodak X-omat AR film (Rochester, NY) for 5–7 d at −80°C with the aid of a Kodak Hi-Plus intensifying screen (Rochester, NY).

Other methods. PKC from mammary gland cytosol was partially purified by diethylaminoethyl (DEAE)-cellulose (DE-52) chromatography (11). PKC from pig spleen was highly purified using ammoniumsulfate fractionation, DE-52, Sephacryl S-200 and protamine-Sepharose 4B chromatography (15). Protein was determined by the method of Bradford (16).

RESULTS

Sphingosine dose-dependently inhibited the activity of PKC obtained by DEAE-cellulose chromatography of mammary gland cytosol (Fig. 1). The IC_{50} value was estimated to be 32 μM, which was similar to the reported value (17). At a higher concentration (417 μM), sphingosine slightly increased both the basal and cofactor-dependent activities. The sphingosine inhibition of PKC was reversed by the addition of higher concentrations of OAG or PS (Fig. 2). Tenfold excess addition of OAG (50 μg/mL) and PS (250 μg/mL) was enough to reverse the inhibition of PKC activity in the presence of 83 μM sphingosine. Reversal by higher concentrations of cofactors of sphingosine inhibition was seen using PKC from different sources and of different purities (Table 1). In the absence of sphingosine, addition of a higher concentration of OAG (50 μg/mL) enhanced PKC activity in both cytosol and the DEAE-cellulose fraction, whereas higher concentrations of PS (250 μg/mL) and Ca^{2+} (250/μM) had little effect on PKC activity. Sphingosine at a concentration of 83 μM was less effective in inhibiting PKC activity in the cytosol than in the DEAE-cellulose fraction. The excess addition of both OAG and PS reversed PKC inhibition in the two fractions, whereas Ca^{2+} was without effect. With 417 μM sphingosine, PKC activity in the two fractions was completely inhibited. However, basal activity was increased, particularly in the cytosol. This increase in basal activity was not observed when highly purified PKC from pig spleen was used in the assay. The increased basal activity of cytosol was restored nearly to the control level by PS, but not by OAG or Ca^{2+}.

The cytosolic and total particulate proteins were phosphorylated in the absence or presence of 10 μM Ca^{2+} and 25 μg/mL PS (Fig. 3). The higher Ca^{2+} concentration was used to fully activate PKC and calmodulin-dependent protein kinase(s). OAG was not included because it was not required for PKC activation at the high Ca^{2+} concentration. Of the phosphoproteins detected, cytosolic 97 kDa phosphorylation was dependent on Ca^{2+}, but not on PS. Its electrophoretic mobility was the same as that of the phosphorylase b used as the marker protein, suggesting that this was phosphorylase b (the substrate for phosphorylase kinase). By comparison, cytosolic 91 kDa, 56 kDa and 38-36 kDa phosphorylation was stimulated by Ca^{2+}, but was more enhanced by the combination with PS. Phosphorylation of cytosolic 72 kDa, 21 kDa and a high molecular weight protein (indicated by the arrow; heavier than myosin heavy chain, 205 kDa), as well as of particulate 91 kDa, 43 kDa, 38 kDa and 36 kDa proteins, showed a more distinct dependency on the two cofactors. Phosphorylation of cytosolic 89 kDa and particulate 56 kDa proteins was considerably enhanced by PS alone. Cytosolic 27 kDa protein was phosphorylated in the absence of the cofactors, but sometimes acted as the PKC substrate (see Figs. 4 and 5). Collectively, these phosphoproteins, except for cytosolic 97 kDa, appeared to be the substrates for PKC. Casein (30 kDa) phosphorylation was independent of the cofactors in the cytosol, but in the particulate fraction were enhanced by PS. The reason for this is unknown at present (the PS-dependency of casein is not apparent in Fig. 6). As described in Materials and

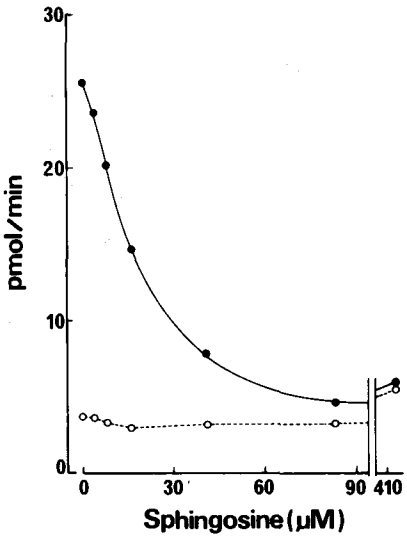

FIG. 1. Dose-dependent inhibition by sphingosine of the activity of protein kinase C partially purified by diethylaminoethyl-cellulose chromatography of the cytosol from bovine mammary gland. The eluant (0.02 mL containing 0.031 mg protein) was assayed in the absence (○) or presence (●) of 5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine and 1 μM Ca^{2+}.

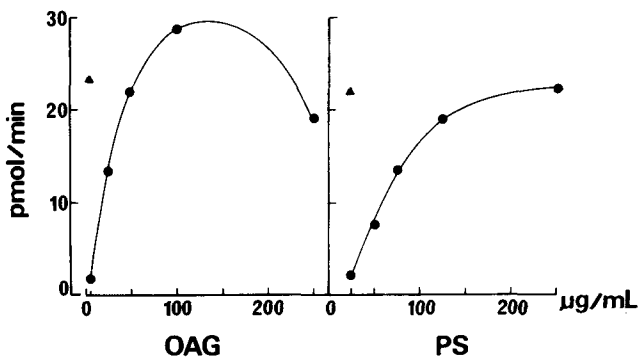

FIG. 2. Reversal by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and phosphatidylserine (PS) of inhibition of protein kinase C (PKC) activity in the eluant from diethylaminoethyl-cellulose chromatography by 83 μM sphingosine. ▲, PKC activity assayed in reaction mixture containing 5 μg/mL OAG, 25 μg/mL PS and 1 μM Ca^{2+}, but not sphingosine.

TABLE 1

Reversal by Higher Concentrations of Cofactors of Sphingosine Inhibition of PKC in the Cytosol, in the Eluant from Diethylaminoethyl (DEAE)-Cellulose Chromatography of the Cytosol and Highly Purified PKC from Pig Spleen

PKC[a]	OAG[b] (μg/mL)	PS[c] (μg/mL)	Ca^{2+} (μM)	Sphingosine (μM)		
				0	83	417
				Protein kinase activity (nmol/min/mg protein)		
Cytosol	0	0	0	0.112	0.149	0.583
Cytosol	5	25	1	0.322	0.205	0.559
Cytosol	50	25	1	0.406	0.352	0.608
Cytosol	5	250	1	0.286	0.275	0.237
Cytosol	5	25	250	0.369	0.220	0.585
DEAE	0	0	0	0.132	0.109	0.189
DEAE	5	25	1	0.864	0.147	0.221
DEAE	50	25	1	1.468	0.581	0.251
DEAE	5	250	1	0.819	0.845	0.318
DEAE	5	25	250	0.864	0.201	0.232
Spleen	0	0	0	2.229	n.d.[d]	2.264
Spleen	5	25	1	9.482	n.d.	2.226

[a]PKC, protein kinase C.
[b]OAG, 1-oleoyl-2-acetyl-*sn*-glycerol.
[c]PS, phosphatidylserine.
[d]n.d., Not determined.

Methods, the homogenate was centrifuged at 38,000 × *g*, instead of 105,000 × *g*. The centrifugation at 38,000 × *g* seemed to reduce the casein contamination of the particulate fraction. Phosphorylation patterns of the 105,000 × *g* supernatant and pellet were essentially the same as those obtained with the 38,000 × *g* fractions (data not shown).

Figure 4 shows the effect of sphingosine on the phosphorylation of the cytosolic proteins in the absence or presence of 5 μg/mL OAG, 25 μg/mL PS and 1 μM Ca^{2+}. Sphingosine inhibited phosphorylation of several proteins. Of the PKC substrates, phosphorylation of the high molecular weight 89, 36 and 21 kDa proteins was inhibited by 83 μM sphingosine. By comparison, 91, 41 and 38 kDa

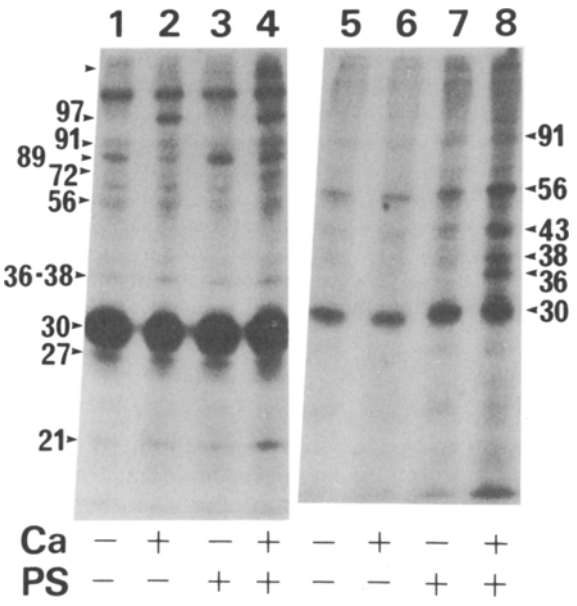

FIG. 3. Phosphorylation of cytosolic (1–4) and total particulate proteins (5–8) of bovine mammary gland in the absence or presence of Ca^{2+} (10 μM) and phosphatidylserine (PS, 25 μg/mL). Endogenous protein kinases in the subcellular fractions were used as the enzyme source.

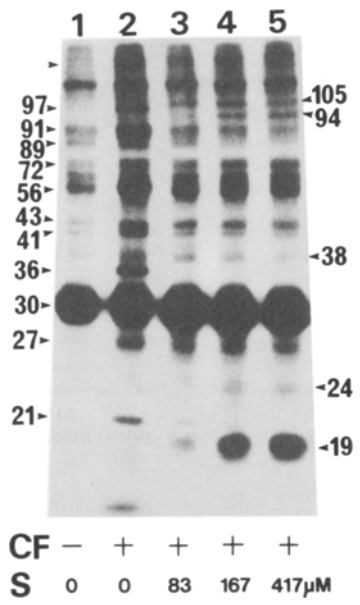

FIG. 4. Autoradiogram showing the effect of sphingosine on the phosphorylation of the cytosolic proteins by protein kinase C. CF, cofactors (5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine and 1 μM Ca^{2+}); S, sphingosine.

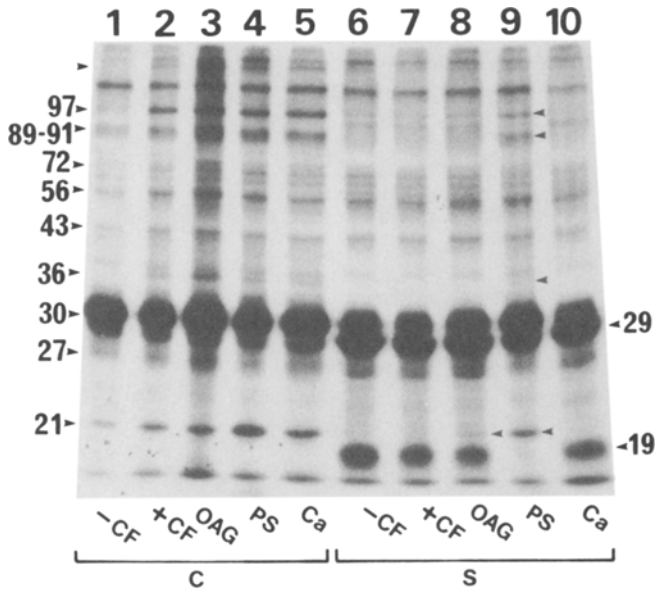

FIG. 5. Reversal by excess addition of the protein kinase C (PKC) cofactors of the cytosolic protein phosphorylation by PKC in the presence of 417 μM sphingosine. C, control (without sphingosine); S, sphingosine; CF, cofactors [5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), 25 μg/mL phosphatidylserine (PS) and 1 μM Ca^{2+}]; OAG, (50 μg/mL OAG, 25 μg/mL PS and 1 μM Ca^{2+}); PS, (250 μg/mL PS, 5 μg/mL OAG and 1 μM Ca^{2+}); Ca, (250 μM Ca^{2+}, 5 μg/mL OAG and 25 μg/mL PS).

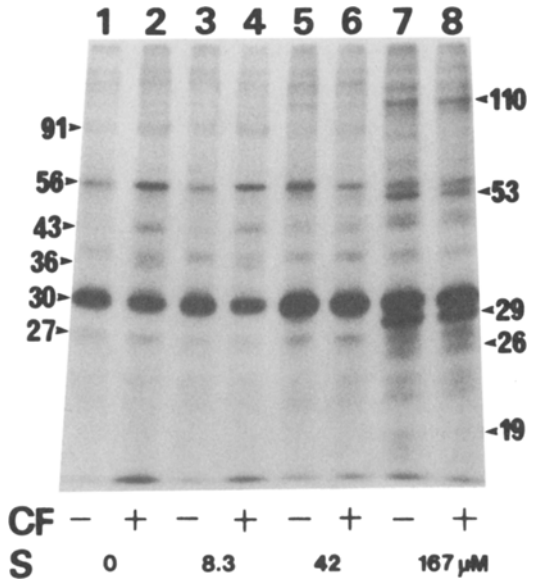

FIG. 6. Effect of sphingosine on total particulate protein phosphorylation by protein kinase C. CF, cofactors (5 μg/mL 1-oleoyl-2-acetyl-*sn*-glycerol, 25 μg/mL phosphatidylserine and 1 μM a^{2+}); S, sphingosine.

phosphorylation was relatively unaffected by sphingosine, because these protein bands were detected, although faintly, even with 417 μM sphingosine. The 72, 56, 43 kDa and 27 kDa phosphorylations appeared to be resistant to sphingosine. The 97 kDa phosphorylation (which was enhanced by Ca^{2+} alone) was effectively inhibited by 83 μM sphingosine. Casein and 135 kDa protein (between the arrow and 97 kDa), whose phosphorylation was independent of the cofactors, were not affected by sphingosine. At higher concentrations of sphingosine (167 and 417 μM), several phosphorylated bands (105, 94, 24 and 19 kDa), which were not detected in the absence of sphingosine (Lane 2), appeared. The bands were not seen when proteins were not included in the reaction mixture, indicating that these bands were not due to artifacts.

The inhibition of the phosphorylation in the cytosol by sphingosine was reversed by excess addition of lipid cofactors (Fig. 5). PS (250 μg/mL) effectively reversed the phosphorylation of 97, 91-89, 36 and 21 kDa proteins (Lane 9). OAG (Lane 8) was less effective in doing so under the conditions used (50 μg/mL in the presence of 417 μM sphingosine), although it enhanced phosphorylation in the absence of sphingosine (Lane 3). Ca^{2+} (250 μM) was without effect (Lane 10). Phosphorylation of 72, 56, 43 and 27 kDa proteins was enhanced by 417 μM sphingosine (compare Lanes 1 and 6). In the presence of cofactors, the effect of sphingosine on 72, 56 and 43 kDa phosphorylation was unclear, irrespective of the cofactor concentrations. However, PS reversed the enhanced 27 kDa phosphorylation nearly to the level seen in Lane 2.

Phosphorylated bands detected at higher concentrations of sphingosine (Fig. 4) can also be seen in Figure 5. Although the 105 kDa band is not clear, 94 kDa (between 97 kDa and 91-89 kDa), 24 kDa (between 27 kDa and 21 kDa) and 19 kDa bands can be seen in the autoradiogram. The appearance of these bands was independent of the cofactors (Lane 6), as in the case of the PKC assay (Table 1). These bands disappeared almost completely upon excess addition of PS (Lane 9), but not upon excess addition of OAG or Ca^{2+}.

In the total particulate fraction, sphingosine suppressed 91 and 36 kDa phosphorylation (Fig. 6). The effect of sphingosine on 56, 43, 38 (between 43 and 36 kDa) and 27 kDa phosphorylation was unclear. Phosphorylated bands that were not detected in the absence of sphingosine were seen with higher concentrations of sphingosine. The 19 kDa band, which was distinct for the cytosol, was faintly seen in the particulate fraction. A 53 kDa band not present in the cytosol, was seen in the particulate fraction, indicating that the band pattern due to sphingosine was different in the cytosol and the particulate fraction. These bands disappeared when excess PS (250 μg/mL) was included (not shown). Phosphorylation of 91 and 36 kDa proteins was also reversed by excess addition of PS, but not by excess addition of OAG or Ca^{2+}.

DISCUSSION

The present study has shown that sphingosine inhibits endogenous protein phosphorylation catalyzed by PKC, as well as PKC activity using histone as the substrate, in bovine mammary gland. The inhibition was overcome by excess addition of lipid cofactors for PKC, particularly PS. To measure PKC activity, the lipid/vesicle assay was used instead of Triton X-100/lipid micellar assay (17). The results obtained with the lipid/vesicle assay were consistent with the phosphorylation patterns shown in autoradiography. For example, the higher basal activity in the cytosol with 417 μM sphingosine (Table 1) was

consistent with the appearance of phosphorylated bands such as the 19 kDa band (Figs. 4 and 5).

The 19 kDa band is similar to that of the 18 kDa protein which is phosphorylated in the presence of 0.4 mM sphingosine in Jurkat T cells (18). The 19 kDa phosphorylation, however, was induced only by pharmacological doses of sphingosine [the band was detected faintly with 83 μM sphingosine (Fig. 4), but not at 42 μM (data not shown)]. Because cellular sphingosine levels in the resting state are in the micromolar range (19), the physiologic significance of the 19 kDa phosphorylation is presently not clear. However, the pathologic importance of the phosphorylation cannot be ruled out, because tumor necrosis factor α, which is increased in inflammation such as mastitis, is known to increase the cellular sphingosine levels (20).

Sphingosine also effectively inhibited phosphorylation of cytosolic 97 kDa protein, and the inhibition was reversed by excess addition of PS. This protein is suggested to be phosphorylase b, because it was phosphorylated by Ca^{2+} alone. The reversal of inhibition by excess PS may be explained by reduced sphingosine binding with the calmodulin subunit of phosphorylase kinase. The inhibition by sphingosine of calmodulin-dependent phosphorylation has already been reported (21). In rat mammary gland, acetyl-CoA carboxylase (240 kDa) and ATP-citrate lyase (116 kDa) have been identified as substrates for PKC (22). The two enzymes are also substrates for calmodulin-dependent multiprotein kinase (22). The high molecular weight substrate (Figs. 3–5) seems to be different from acetyl-CoA carboxylase, because its phosphorylation was not enhanced by Ca^{2+} alone.

PKC substrates can be classified into two groups with respect to the mode of modulation by sphingosine: one is inhibited at both lower and higher concentrations and includes the 36 kDa protein in both the cytosolic and particulate fractions; the other group containing cytosolic 72 kDa protein is resistant to inhibition by sphingosine at lower concentrations, and its phosphorylation is enhanced at higher concentrations in the absence of the cofactors. The different sensitivity may be explained by the assumption that the substrates in the two groups are catalyzed by different PKC isozymes. Another possibility is that sphingosine directly interacts with the substrate proteins. Since sphingosine interacts with phospholipids (7,8) and inhibition of substrate phosphorylation by sphingosine is overcome by PS, it is conceivable that sphingosine binds to phospholipid-binding and Ca^{2+}-binding proteins such as annexins (23). Mammary gland contains proteins of the annexin family, including calelectrin (70 kDa) and calpactin (36 kDa) (24). Our preliminary study suggests that the 36 kDa protein in both fractions is annexin I because the protein reacted with anti-annexin I as shown by immunoblot analysis. Identification of substrates for PKC as well as for calmodulin-dependent protein kinase(s) is required for further study of the physiologic and pathologic importance of sphingosine in bovine mammary gland function.

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Intravenous Injection of Tridihomo-γ-linolenoyl-glycerol into Mice and Its Effects on Delayed-Type Hypersensitivity

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Highly purified tridihomo-γ-linolenoyl-glycerol (DGLA-TG) was emulsified with egg yolk lecithin as a 10% (wt/vol) DGLA-TG emulsion. We injected 0.05 or 0.5 mL of the emulsion into mice through the tail vein and investigated its effects on the fatty acid composition of spleen cells and on delayed-type hypersensitivity (DTH) response. At 1 h after the injection, dihomo-γ-linolenic acid (DGLA) concentrations were increased significantly in the total phospholipid fraction of spleen cells from 1.21 ± 0.13 mol% to 2.09 ± 0.74 mol% ($P < 0.02$) and 7.95 ± 1.25 mol% ($P < 0.001$) in the 0.05-mL and 0.5-mL groups, respectively. Mice, which had already been immunized with sheep red blood cells (SRBC), were challenged by the injection of SRBC into the right-hind footpad. Intravenous injection into mice with 0.5 mL of the emulsion immediately before the challenge almost completely suppressed DTH response measured by the swelling of the right-hind footpads 24 h thereafter. This inhibitory effect on the DTH response was significant with as little as 0.05 mL of the emulsion, whereas a soybean oil emulsion was not effective at all. In conclusion, intravenous injection of a DGLA emulsion increased DGLA concentrations in immune cells within 1 h and suppressed the DTH reaction.
Lipids 28, 873–876 (1993).

Dihomo-γ-linolenic acid (20:3n-6, DGLA) is an elongation product of γ-linolenic acid (18:3n-6, GLA). DGLA is further converted to arachidonic acid (20:4n-6, AA) through Δ5 desaturation. DGLA is also the precursor fatty acid of prostaglandin E_1 (PGE_1), which has immunosuppressive effects through increase in intracellular cyclic adenosine monophosphate (AMP) levels (1). *In vitro* administration of DGLA depresses interleukin-2 (IL-2) production by human peripheral mononuclear lymphocytes (2) and the proliferation of human synovial cells also by increasing their cytosolic cyclic AMP levels (3). In addition, oral administration of DGLA has been shown to depress human platelet aggregation (4–6).

Delayed-type hypersensitivity (DTH) response is one of the T cell-mediated immune responses (7) and is probably related to allograft rejection. In the present study we formulated an injectable emulsion of DGLA and examined the effect of its injection on DTH response. We also checked how quickly injection of the emulsion changed the fatty acid composition of mouse spleen cells and plasma.

MATERIALS AND METHODS

Materials. Female BALB/c mice (6–8 wk of age) were purchased from Sankyo Labo Service (Tokyo, Japan). They were housed in groups of five in a temperature-, light- and humidity-controlled room. They were fed standard chow and tap water *ad libitum* throughout the experiments. Tridihomo-γ-linolenoyl-glycerol (DGLA-TG) (purity >95%) was kindly donated by Idemitsu Petrochemical Co. (Tokyo, Japan) and emulsified according to the method of Geyer *et al.* (8). One hundred mL of the emulsion contained 10 g of DGLA-TG, 1.2 g egg yolk lecithin (Asahi Chemicals, Tokyo, Japan) as an emulsifier and 2.5 g glycerol. A 10% soybean oil emulsion (Intralipid) was generously donated by Otsuka Pharmaceuticals (Tokyo, Japan). Sheep red blood cells (SRBC) purchased from Nippon Bio-Sup Center (Tokyo, Japan) were washed three times and resuspended in sterilized saline just before use.

Measurement of DTH response. Mice were primed by subcutaneous injection of 0.1 mL of a 10% SRBC suspension. Six days later, 50 μL of a 20% SRBC suspension was injected into the right-hind footpad. Mice were injected with 0.05 or 0.5 mL of the DGLA-TG emulsion, 0.5 mL of Intralipid, or 0.5 mL of a 2.5% glycerol solution through the tail vein, immediately before the second injection of SRBC. In order to inject 0.05 mL of the emulsion, 0.5 mL was injected after the emulsion had been diluted 10 times with the glycerol solution.

Twenty-four hours after the second injection of SRBC, the dorsoventral thickness of the right- and left-hind footpads of each mouse was measured three times with a vernier caliper. The mean difference between both footpads was taken as the DTH response for each mouse.

Fatty acid analysis. A separate set of experiments was used for fatty acid analysis. Mice were injected similarly with 0.05 or 0.5 mL of the DGLA-TG emulsion without any SRBC injection. The fatty acid composition of the spleen cell phospholipid (PL) and plasma free fatty acid (FFA) fractions was analyzed before the injection (0 h), and 1 and 6 h thereafter. Five mice were used at each time point and for each dose.

Ethylenediaminetetraacetic acid-anticoagulated blood samples were taken from the retroorbital plexus under diethyl ether anesthesia, and plasma was obtained by centrifugation. Spleens were also obtained immediately after the blood sampling. Each spleen was minced through a stainless steel mesh and suspended in cold Hank's balanced salt solution without phenol red (HBSS; Whittaker Bioproducts, Walkersville, MD). Contaminating red blood cells were lysed with a 34 mM *tris*(hydroxymethyl)aminomethane-NH_4Cl buffer (pH 7.4), and spleen cells were washed three times with HBSS. Plasma and spleen cells were stored at −80°C until fatty acids were analyzed.

The fatty acid composition of spleen cells and plasma was analyzed as follows: The total PL fraction of spleen cells and the FFA fraction of plasma were separated by thin-layer chromatography (TLC) on Silica Gel 60 plates (5 × 20 cm, 0.25 mm thick; Merck, Darmstadt, Germany) after total lipid extraction with chloroform/methanol (2:1, vol/vol). After transmethylation with 6% sulfuric acid/methanol, fatty acid methyl esters of each fraction were analyzed using a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an SP-2330 capillary

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Abbreviations: AA, arachidonic acid; AMP, adenosine monophosphate; ANOVA, analysis of variance; DGLA-TG, tridihomo-γ-linolenoyl-glycerol; DGLA, dihomo-γ-linolenic acid; DTH, delayed-type hypersensitivity; FFA, free fatty acid; GLA, γ-linolenic acid; HBSS, Hank's balanced salt solution; IL-2, interleukin-2; LT, leukotriene; PL, phospholipid; PG, prostaglandin; SRBC, sheep red blood cell; TLC, thin-layer chromatography.

column (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA). The temperature of the injection port was 250°C. Column temperature was kept at 160°C for 10 min, then raised from 160 to 200°C at 4°C/min and then held at 200°C. The detection was done by flame-ionization. Helium was used as a carrier gas at an inlet pressure of 0.56 kg/cm^2. Peaks were identified by comparison of retention times with those of authentic fatty acid standards (Sigma Chemical Co., St. Louis, MO).

Statistics. The results are expressed as means ± SD. The DTH response of the emulsion-injected groups was compared with that of the vehicle-injected group by an unpaired *t*-test with Bonferroni's adjustment after analysis of variance (ANOVA). Fatty acid concentrations after DGLA injection were similarly compared with those before the injection. In the case of spleen cells, Wilcoxon 2-sample test was also performed with Bonferroni's adjustment. In the case of plasma-free DGLA concentrations, data were log-transformed and compared using the unpaired *t*-test with Bonferroni's adjustment after ANOVA. $P < 0.05$ was taken as significant.

RESULTS

As shown in Figure 1, the injection of the DGLA-TG emulsion immediately before the second injection of SRBC markedly suppressed DTH response as compared with vehicle injection, whereas Intralipid injection did not alter the response at all. The effect of the DGLA-TG emulsion was dose-dependent, and the DTH response was significantly inhibited, even with 0.05 mL of the DGLA-TG emulsion (Fig. 1).

Vehicle injection did not change the fatty acid composition of either spleen cells or plasma (data not shown); therefore, for simplicity's sake, we compared the fatty acid composition before and after DGLA injection instead of comparing data after DGLA and vehicle injection at each time point and at each dose. Changes in the fatty acid composition of the spleen cell PL fraction after DGLA injection are shown in Table 1. DGLA concentrations were significantly increased both 1 and 6 h after the injection at either dose. AA concentrations were slightly, but significantly, decreased 1 h after the injection in the 0.5-mL group only.

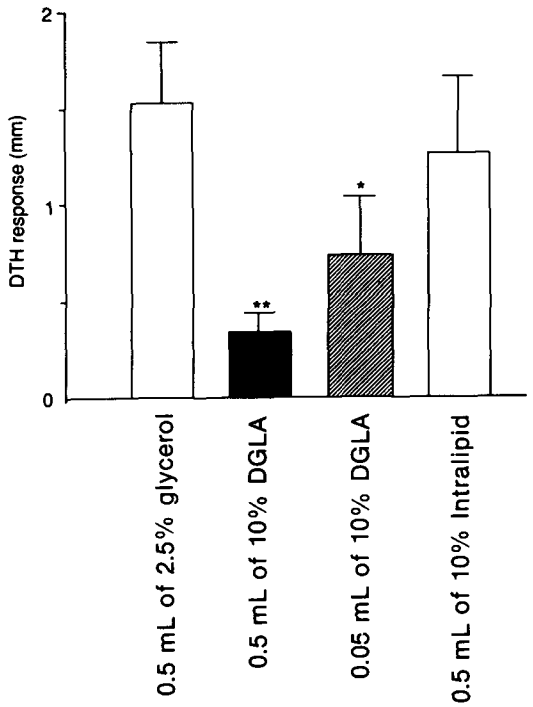

FIG. 1. The effect of a dihomo-γ-linolenic acid (DGLA) emulsion and a soybean oil emulsion (Intralipid) on delayed-type hypersensitivity (DTH) response. The effect of intravenous administration of a 10% tridihomo-γ-linolenoyl-glycerol emulsion on DTH response to sheep red blood cells (SRBC) was compared with that of a 2.5% glycerol solution. For this purpose, 0.05 mL or 0.5 mL of the DGLA emulsion, 0.5 mL of 10% Intralipid, or 0.5 mL of the glycerol solution was injected through the tail vein immediately before the second challenge of SRBC into the right-hind footpad. Twenty-four hours later the thickness of both hind footpads was measured three times. A mean difference (in mm) between the right and left footpad thickness was regarded as the DTH response. The DTH response of each group is expressed as mean ± SD from five mice. Significant differences from the control group injected with 0.5 mL of the glycerol solution are shown by *$P < 0.01$ and **$P < 0.001$.

Changes in DGLA concentrations in the plasma FFA

TABLE 1

Changes in Fatty Acid Composition (mol %) of the Spleen Cell PL Fraction after DGLA Injection[a]

	0 h	0.05-mL injection		0.5-mL injection	
	(n = 5)	1 h (n = 5)	6 h (n = 5)	1 h (n = 5)	6 h (n = 5)
16:0	26.94 ± 0.76	27.68 ± 2.54	24.91 ± 0.53	26.55 ± 3.79	28.52 ± 5.05
18:0	15.60 ± 0.72	17.10 ± 0.30[e]	15.33 ± 0.49	14.44 ± 1.65	16.10 ± 1.03
18:1n-9	6.30 ± 0.49	6.50 ± 0.56	6.77 ± 0.35	6.52 ± 0.45	6.52 ± 0.32
18:1n-7	2.87 ± 0.15	2.98 ± 0.12	2.83 ± 0.24	2.76 ± 0.22	3.01 ± 0.18
18:2n-6	7.69 ± 0.39	7.99 ± 0.12	7.99 ± 0.37	7.74 ± 0.57	8.11 ± 0.45
20:3n-9	1.10 ± 0.12	0.98 ± 0.35	1.04 ± 0.06	0.90 ± 0.12	1.06 ± 0.10
20:3n-6	1.21 ± 0.13	2.09 ± 0.74[b]	2.08 ± 0.24[c]	7.95 ± 1.25[e]	2.06 ± 0.40[f]
20:4n-6	17.42 ± 0.60	16.55 ± 0.48	17.51 ± 0.80	15.69 ± 0.49[d]	16.86 ± 1.08
22:4n-6	1.71 ± 0.10	1.66 ± 0.08	1.75 ± 0.12	1.30 ± 0.73	1.60 ± 0.24
22:5n-3	1.84 ± 0.10	1.90 ± 0.23	1.94 ± 0.13	1.57 ± 0.26	1.80 ± 0.34
22:6n-3	7.29 ± 0.44	6.89 ± 0.31	7.36 ± 0.54	6.56 ± 0.47	6.56 ± 0.87

[a]Mice were injected with 0.05 or 0.5 mL of a 10% dihomo-γ-linolenic acid (DGLA) emulsion through the tail vein. Spleen cells were obtained 1 or 6 h after the injection and analyzed for fatty acid composition of their total phospholipid (PL) fraction by gas chromatography. Fatty acids, which comprised more than 1%, are listed. [b-f]Data were compared with the 0-h group; [b]$P < 0.02$; [c]$P < 0.01$; [d]$P < 0.005$; [e]$P < 0.001$ by unpaired *t*-test with Bonferroni's adjustment; and [f]$P < 0.05$ by Wilcoxon 2-sample test with Bonferroni's adjustment.

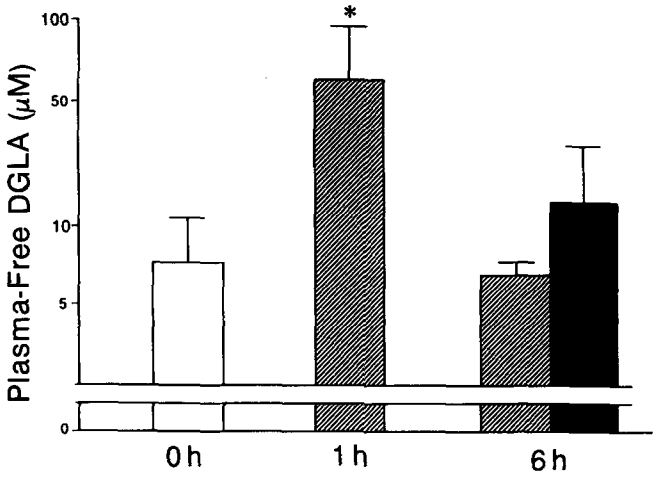

FIG. 2. Comparison of plasma free dihomo-γ-linolenic acid (DGLA) concentrations before (the 0-h group, white column) and after the injection of 0.05 mL (grey columns) or 0.5 mL (black column) of a 10% tridihomo-γ-linolenoyl-glycerol emulsion. Data were log-transformed. Significant difference from the 0-h group is indicated by *$P < 0.01$.

fraction are shown in Figure 2. The concentrations were significantly increased 1 h after the injection in the 0.05-mL group. We could not successfully analyze the plasma FFA fraction 1 h after the injection in the 0.5-mL group because the excessive amount of DGLA-TG present in plasma samples made it difficult to separate FFA from DGLA-TG by TLC.

DISCUSSION

A number of studies have been concerned with the effects of orally administered DGLA in animals (4,6) and in humans (4,5,9). However, the effect of intravenously injected DGLA, except for our recent work (10), had not been studied previously.

As shown in Figure 1, DTH response was suppressed by a single injection of a DGLA emulsion immediately before the second SRBC injection. The mechanism of action of DGLA is not clear at present, although conceivable mechanisms are discussed below.

The plasma-free DGLA concentrations were drastically increased to about 50 μM at 1 h after the injection of 0.05 mL of the DGLA emulsion. Taking into account that the ID_{50} concentrations of DGLA for IL-2 production are about 30 μm (2), IL-2 production around the SRBC-injected footpad appears to be reduced in the 0.05-mL group. The rate of reduction in IL-2 production may be even greater in the 0.5-mL group than in the 0.05-mL group. Because Th1 clones, which are significantly involved in DTH in mice (7), proliferate in response to IL-2 (11), the reduction in IL-2 may be at least partly involved in the anti-inflammatory action of DGLA.

DGLA is a substrate of cyclooxygenase, and the rate of conversion of DGLA to E-series PG (PGE_1) is greater than that of AA to PGE_2 (12). Actually, oral administration of DGLA resulted in increased PGE_1 output by a renal papilla preparation of rabbits (13) and human platelet-rich plasma (5). PGE_1 is a well-known immunosuppressant (1) and depresses the expression of the class II histocompatibility complex (Ia molecules) (14). On the other hand, the conversion of DGLA to thromboxane (TX), which is an antagonist of PGE_1 in terms of immune response (14), was reported to be negligible (15,16). Also, the effect of TXA_1 synthesized from DGLA was reported to be much less pronounced than that of TXA_2 (15). Moreover, *in vivo* DGLA administration inhibits TXA_2 production (17). Taken together, DGLA appears to be a potent immunosuppressive fatty acid.

In addition to cyclooxygenase products, lipoxygenase products are also involved in immune response (18). Due to the absence of a double bond at C-5, DGLA is not a substrate of 5-lipoxygenase (19), and thus leukotrienes (LT) are not synthesized from DGLA except for the isomer of LTC_3 that is oxygenated at C-8 (20). We recently observed that the intravenous administration of the same DGLA emulsion used in the present study reduced LTB_4 production by rat peritoneal resident macrophages within 6 h by one-half (10). Guichardant *et al.* (21) also observed that supplementation with DGLA inhibited LTB_4 production by human leukocytes *in vitro*. Because LTB_4 augments IL-1 production (22) and complements IL-2 activity (23), it is conceivable that the inhibitory effect on LTB_4 production is one of the mechanisms for suppression of DTH response. LTB_4 may be involved in any of the steps of DTH response with or without intervening in cytokine production.

The increased free DGLA levels in plasma after DGLA injection probably affect the PL fatty acid composition of local lymph nodes near footpads. In preliminary experiments we tried to determine the fatty acid composition of local lymph nodes. However, the nodes were too small to yield reliable fatty acid compositional data. Instead of measuring the fatty acid composition of lymphocytes of the local lymph nodes, we determined the fatty acid composition of the PL fraction of spleen cells. As shown in Table 1, DGLA was quickly increased in both the 0.05-mL and 0.5-mL groups. It is likely that similar changes took place in local lymphocytes affecting lymphocyte function.

Horrobin (24) has reported the effects of evening primrose oil containing GLA. The mechanism of the effect of GLA administration is probably through the elongation of GLA to DGLA. It also takes a few weeks upon oral administration of GLA to observe an effect (24). In this case, intravenous injection of DGLA seems superior to that of primrose oil. Emulsified lipids are also taken up preferentially by inflammatory tissues (25), which helps in the accumulation of DGLA in those tissues in which DGLA takes effect.

If in the future DGLA emulsions should clinically be applied to patients that require a rapid correction of DTH response, as is the case in allograft rejection, the present observations could provide basic information about the doses required and the pharmacokinetics of such emulsions.

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pH-Dissociation Characteristics of Cardiolipin and Its 2′-Deoxy Analogue

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Cardiolipin (CL) is found in inner mitochondrial membranes and the plasma membrane of aerobic prokaryotes. CL is tightly bound to those transmembrane enzymes associated with oxidative phosphorylation. CL has earlier been reported to have a single pK at low pH. We have titrated CL in aqueous suspension (bilayers) and in solution in methanol/water (1:1, vol/vol) and found it to display two different pK values, pK_1 at 2.8 and pK_2 initially at 7.5 but shifting upwards to 9.5 as the titration proceeds. The unusually high pK_2 might be explained by the formation of a unique hydrogen bond in which the free hydroxyl on the central glycerol forms a cyclic intramolecular hydrogen-bonded structure with one protonated phosphate (P-OH group). We have therefore chemically synthesized the 2′-deoxycardiolipin analogue, which lacks the central free hydroxyl group, and measured its pH-dissociation behavior by potentiometric titration, under the same conditions as those for CL. The absence of the hydroxyl group changes the titration dramatically so that the deoxy analogue displays two closely spaced low pK values (pK_1 = 1.8; pK_2 = 4.0). The anomalous titration behavior of the second dissociation constant of CL may be attributed to the participation of the central glycerol OH group in stabilizing the formation of a cyclic hydrogen-bonded monoprotonated form of CL, which may function as a reservoir of protons at relatively high pH. This function may have an important bearing on proton pumping in biological membranes.

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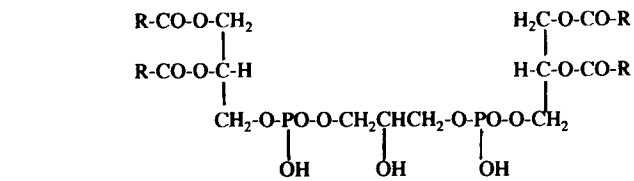
Almost a century ago, Wasserman *et al.* (1) reported the finding of "specific" antibodies in the sera of syphilitic patients by using the antigen in alcohol extracts from the spleen of a syphilitic fetus. Within a few years, Browning *et al.* (2) established that the antigen could be extracted from a variety of tissues, and Noguchi (3) discovered that the ether-soluble antigen was precipitable by acetone, a characteristic of phospholipids. The latter observation provoked Pangborn (4) to isolate and purify the antigen from beef heart by means of the Wasserman antibody assay. She named the isolated antigen "cardiolipin" (CL) and subsequently chemically characterized it as an acylated polyglycerolphosphate complex (5). CL was soon found to be present not only in mammals but in bacteria and plants (6-8).

Although CL was one of the first membrane lipids to be isolated in pure form, its structure proof and synthesis were not achieved until the 1960s. Le Cocq and Ballou (9) established its structure and configuration as di-*O*-(*sn*-3-phosphatidyl)-*sn*-1′,3′-glycerol (Fig. 1). The total synthesis of CL was soon achieved by de Haas *et al.* (10).

Few *et al.* (11), in a classic paper in which they measured pK values of surface charges of suspensions of bacterial cells and cell lipid extracts by microelectrophoresis, determined the pK_a of a sample of CL obtained from Pangborn. The pK_a of CL they reported was 1.05. They also found that the lipids of *Micrococcus lysodeikticus* displayed a pK_a of 0.4. A curious feature of the titration curves obtained by Few *et al.* (11) was that unlike those of the *M. lysodeikticus* cells and the lipids, the CL titration continued to rise after the observed endpoint.

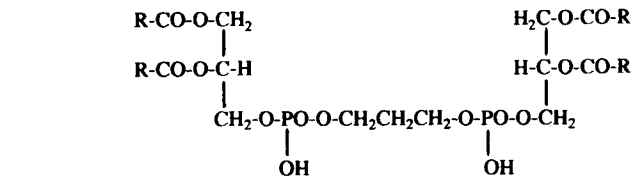
Shortly thereafter, Coulon-Morelec *et al.* (12) also reported a single pK below pH 4.0 for CL based on titration in ethanol solution using indicator dyes. In a more recent study, Seddon *et al.* (13) reported a pK of 2.8 based on the pH dependence of phase changes. In all three measurements, it was assumed that there is only one pK for the two phosphate protons, presumably because the molecule is symmetrical. This view is widely held in the literature (14-16).

CL has a variety of unique physical and chemical properties. Unlike most phospholipids, it readily forms a hexagonal II(H_{II}) phase (17), which is easily characterized by ^{31}P nuclear magnetic resonance (NMR) (18). This



RCO = 18:0

Hydrogenated cardiolipin (CL)



RCO = 16:0

2′-Deoxycardiolipin (dCL)

FIG. 1. Chemical structures of CL and dCL.

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Abbreviations: BH, beef heart; CL, cardiolipin (diphosphatidylglycerol); CL-BH, cardiolipin from beef heart; 18:0-CL (H_2-CL-BH), hydrogenated beef heart cardiolipin; H_2-CL-EC, hydrogenated cardiolipin from *E. coli; cy*-17:0 and *cy*-19:0, 9,10-methylenehexadecanoic and octadecanoic acid, respectively; DAG, diacylglycerols; dCL, 2′-deoxycardiolipin (diphosphatidyl-1,3-propanediol); 16:0-dCL, synthetic deoxycardiolipin with palmitoyl (16:0) as acyl groups; DPG, dipalmitoylglycerol; DPPA, dipalmitoyl phosphatidic acid; FAB-MS, fast-atom bombardment mass spectrometry; FTIR, Fourier transform infrared spectroscopy; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PPD, phosphatidylpropanediol; PPOP, phosphatidylpropanediol-phosphate; TLC, thin-layer chromatography.

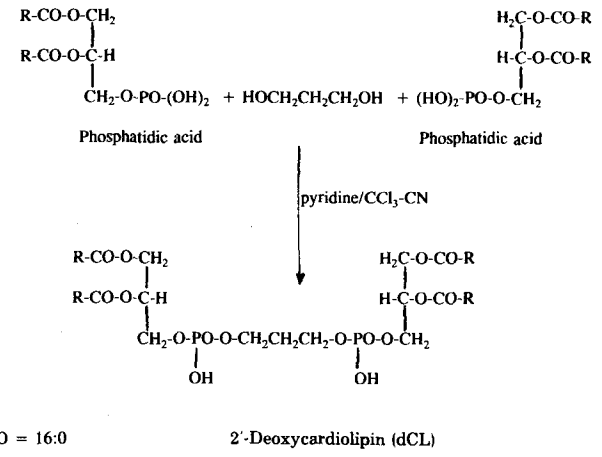

FIG. 2. Scheme for the synthesis of 2'-deoxycardiolipin.

characteristic is promoted by a fatty acid chain composition rich in polyunsaturated fatty acids, as is found in mitochondrial CL. A lesser known and more poorly understood chemical quality is the low degree of accessibility of the free hydroxyl group to acetylation in pyridine using vigorous conditions (19,20). In this connection, recent Fourier transform infrared (FTIR) studies of CL have shown (21) that the central free hydroxyl group can form intramolecular hydrogen bonds with the phosphate groups, which might explain the difficulty in acetylating the C-OH group.

In mitochondria, CL is located largely on the matrix side of the inner membrane (for a review, see Ref. 22). CL is known to bind tightly to inner mitochondrial membrane-bound enzymes, such as cytochrome oxidase (23), the F_0F_1ATPase (24), and the ADP/ATP exchange protein (25), and to play an essential role in the optimal functioning of cytochrome c oxidase (26) and F_0F_1-ATPase (27,28). Nonetheless, the specific role of this lipid still requires clarification.

Knowledge of the pH-dissociation characteristics of CL may be of interest in connection with its biological structure-function relationships, particularly that of a proton reservoir in energy transducing membranes. We therefore examined the pH potentiometric titration of CL and found that, instead of titrating as a simple dibasic acid, it displayed two widely separated pK values, one at 2.8 and a second, anomalous pK above physiological pH. In order to explain the existence of the second pK, we synthesized 2'-deoxycardiolipin (dCL) (Figs. 1 and 2). This permitted us to examine the role of the central free hydroxyl group of the connecting glycerol in the anomalous CL titration. The dCL titrated as a typical dibasic acid with two closely spaced pK values below pH 4. Comparison of the titration behavior of dCL and CL suggests that the free hydroxyl of the connecting glycerol in CL participates in stabilizing the monoprotonated form of the CL molecule, thus enabling it to function as a proton reservoir at high pH (see Discussion section).

MATERIALS AND METHODS

Chemicals. All solvents were glass distilled prior to use, except for pyridine and triethylamine, which were dried over fresh potassium hydroxide pellets and used without distillation. Dipalmitoyl phosphatidic acid (DPPA, disodium salt) and *Escherichia coli* CL [Na salt; fatty acid analysis by gas-liquid chromatography (GLC): 16:0, 43.0%; *cy*-17:0, 24.2%; *cy*-19:0, 20.6%; 14:0, 1.4%; 16:1, 1.6%; 18:0, 1.6%; 18:1, 5.2%] were purchased from Sigma Chemical Co. (St. Louis, MO); beef heart (BH) CL (Na salt) was purchased from Sigma (fatty acid analysis by GLC: 18:1, 6.7%; 18:2, 92.2%; 16:0, 0.5%; 16:1, 0.4%; 18:0, 0.2%) or from Avanti (Pelham, AL). Adams' catalyst ($PtO_2 \cdot xH_2O$, Gold label), 1,3-dibromopropane and trichloroacetonitrile were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Hydrogenation of CL. CL-BH (*ca.* 25 mg) or *E. coli* CL (*ca.* 13 mg) were hydrogenated in methanol (10 or 5 mL, respectively) in the presence of 170 or 100 mg, respectively, of Adams' catalyst in an all-glass hydrogenator by stirring in an atmosphere of hydrogen gas at room temperature (22-24°C) until uptake of hydrogen ceased (*ca.* 2-3 h). The mixture was centrifuged, and the platinum catalyst washed twice with 1 mL of chloroform/methanol (1:1, vol/vol). The combined supernatants were diluted with the appropriate amounts of chloroform and 0.2N HCl (final ratio, chloroform/methanol/water, 1:2:0.8, by vol) to form a biphasic system (29); the chloroform layer was removed, neutralized by addition of 1 N ammonium hydroxide in methanol, diluted with benzene and concentrated to a small volume under a stream of nitrogen. The ammonium salt of hydrogenated CL was then precipitated by the addition of 10 vol of acetone and cooling at −10°C; it was collected by centrifugation, reprecipitated from chloroform by addition of acetone, washed with cold acetone and finally dried *in vacuo;* the yield of hydrogenated CL was 80-85%; thin-layer chromatography (TLC) R_f, 0.48 in solvent A (R_f of unhydrogenated CL, 0.48). Fatty acid analysis by GLC: BH hydrogenated CL (H_2-CL-BH): 14:0, 1.0%; 16:0, 9.3%; 16:1, 2.5%; 18:0, 76.6%; 18:1(*t*), 9.4%. *E. coli* hydrogenated CL (H_2-CL-EC): 14:0, 2.8%; 16:0, 50.5%; *cy*-17:0, 19.1%; 18:0, 8.9%; *cy*-19:0, 12.9%. Positive fast-atom bombardment mass spectrometry (FAB-MS) of H_2-CL-EC showed major ion peaks at 523, 551, 563, 579, 591 and 607 *m/z,* corresponding, respectively, to 14:0/14:0, 16:0/16:0, 16:0/*cy*-17:0, 16:0/18:0, 16:0/*cy*-19:0 and 18:0/18:0 molecular species of diacylglycerols (DAG), consistent with the fatty acid analytical data. Negative FAB-MS did not yield any diagnostic ion peaks.

Chromatography. TLC of CLs and the deoxy analogue was carried out on 20 × 20 cm plates coated with silica gel 60A K6 (0.25 mm thick; Whatman International Co., Maidstone, England) using chloroform/methanol/conc. (14M) ammonium hydroxide (65:35:5, by vol) as solvent (Solvent A). GLC of fatty acid methyl esters, prepared by methanolysis of CL or dCL samples in methanolic HCl (30), was carried out on a column of SP-2330 (Supelco, Inc., Bellefonte, PA) at 185°C on a PYE Unicam (Cambridge, England) gas chromatograph.

Physical measurements. ^1H and ^{13}C NMR spectra were measured in $CDCl_3$ on a Bruker (Karlsruhe, Germany) CXP-300 spectrometer. FAB-MS was carried out on a VG-7070E (Vacuum Generators, Inc., Manchester, England) mass spectrometer with digital RL 02 data system and printonic printer.

Potentiometric titration. Samples (6-15 mg, 4-11 μmoles) of H_2-CL-BH, H_2-CL-EC, or 16:0-dCL, were converted to their respective protonated form using an

acidified Bligh and Dyer (29) procedure (see Ref. 30) and suspended in 2 mL of 0.05 M KCl by vortexing and sonication with a needle tip sonicator (Sonic Dismembranator, Quigley-Rochester, Inc., Rochester, NY). The samples of free acids formed milky-to-translucent dispersions in 0.05 M KCl, which were titrated with 0.05N KOH in 0.05 M KCl using a glass electrode, accurate over the pH range 2-12, and a pH meter (Radiometer, Copenhagen, Denmark). The KOH solution was delivered in increments with a syringe microburet (1 mL; Micro-Metric Instrument Co., Cleveland, OH). In some experiments, samples were sonicated in a cup horn type sonicator (Ultrasonics, Inc., Model W-385, Plainview, NY), with a Lauda K-2/R water circulator (Brinkmann Instruments, Westbury, NY), and titrated with an Orion digital pH meter (Model 601A) with a microelectrode (Microelectrodes Inc., Model No. MI-410, Londondery, NH), using a Hamilton syringe (2 mL) and a syringe pump (Razel Scientific Instruments, Stamford, CT) for constant delivery of the aqueous 0.05M KOH.

Titrations were also done on solutions of the protonated forms of CL or dCL (4-9 μmoles) in 2 mL of methanol/aqueous 0.05 M KCl (1:1, vol/vol). It was difficult to suspend the free acid form of the natural, unsaturated BH-CL in water by sonication and vortexing. This lipid was therefore titrated only in methanol/0.05 M KCl (1:1, vol/vol).

All titrations were done at room temperature (22-24°C) and care was taken to minimize exposure of samples to pH conditions above 8. Total consumption of alkali was in the range of 95-105 and 85-100% of the theoretical values calculated for CL and dCL, respectively. Immediately after titration, samples were recovered (85-100%, depending on the fatty acid composition) by acetone precipitation of the ammonium salt form (30); little or no degradation was observed by TLC using Solvent A.

Titration curves were computer-simulated using the general equation (31) for either monoprotic or diprotic systems. For the CL titrations, the curve after the first endpoint was calculated according to the approximation:

$$pH = pK_a(eff) + \log\{[A^-]/[HA]\} \quad [1]$$

where $pK_a(eff)$, the effective pK_a, varies as the titration of CL proceeds. In this case, it is not a constant as it is in the general equation (31). The theoretical curve for dCL was calculated on the basis of the equation:

$$V_b = \frac{V_a\,\alpha\gamma - V_b\,\beta F_a + F_a V_a \alpha}{\alpha(F_b\gamma)} \quad [2]$$

where V_b = volume of base added; V_a = initial volume of acid; F_a = initial formality of the acid; F_b = normality of base; $\alpha = (H^2)/(K_1K_2) + (H)/(K_2) + 1$; $\beta = (H^2)/(K_1K_2) - 1$; and $\gamma = (K_W)/(H) - H$; $H = [H^+]$ and K_1 and K_2 = the first and second dissociation constants of a diprotic acid.

The water-soluble phosphate esters, glycerol-1,3-diphosphate and 1,3-propanediol-1,3-diphosphate, were titrated in water as described for CL and dCL.

Syntheses of glycerol-1,3-diphosphate. Glycerol-1,3-diphosphate was synthesized as described previously (32) by condensation of 1,3-dibromo-2-palmitoyloxypropane with silver dibenzylphosphate (33) followed by removal of the benzyl groups by catalytic hydrogenolysis (Pd/C) and the palmitoyl group by mild alkaline hydrolysis; the product was isolated as the dibarium salt and converted to the free acid by treatment with Dowex-50 (H^+) ion-exchange resin and to various salt forms by neutralization with the appropriate base (32).

Synthesis of 1,3-propandiol-1,3-diphosphate. 1,3-Propandiol-1,3-diphosphate was synthesized by a procedure analogous to that for glycerol-1,3-diphosphate, in which 1,3-dibromopropane (0.377 g, 1.87 mmol) was condensed with silver dibenzylphosphate (1.4 g, 3.6 mmol) in anhydrous benzene (13.5 mL) under reflux with stirring for 24 h, followed by hydrogenolysis of the benzyl groups of the blocked product in ethanol (4 mL) with Pd/C catalyst (200 mg). The final product was isolated as the dibarium salt (28.4 mg, 0.055 mmol; 3% yield) and converted to the free acid form and to other salt forms as described for glycerol-1,3-diphosphate (32).

Synthesis of 2'-deoxy-cardiolipin (16:0-dCL). The synthesis of 16:0-dCL was carried out as summarized in Figure 2. DPPA disodium salt (67 mg, 0.1 mmol) was converted to the free acid form (30), dried in a desiccator over KOH pellets, mixed with an excess of 1,3-propanediol (140 mg, 1.84 mmol) and dissolved in dry pyridine or triethylamine (10 mL) at 50°C. The condensation was then brought about by adding trichloroacetonitrile (4 mL) to the clear tan solution and stirring in a nitrogen atmosphere at 50°C for 20 h (34). The dark brown mixture was diluted with an equal volume of benzene, concentrated in a rotary evaporator at 40-50°C and dried in a desiccator over concentrated sulfuric acid and then KOH pellets. The black residue was dissolved in 12 mL of chloroform/methanol (1:1, vol/vol), diluted with 5.4 mL of 1 N HCl and the mixture was centrifuged. The chloroform layer was removed, washed with an equal volume of methanol/water (10:9, vol/vol), made alkaline with 0.2 N ammonium hydroxide in methanol and concentrated to dryness *in vacuo*. The residue was dissolved in 0.5 mL of chloroform, diluted with 5 mL of acetone, and cooled on ice. The black precipitate of crude product, 16:0-dCL, was centrifuged, washed with cold acetone and reprecipitated from a small volume of chloroform (0.5 mL) by the addition of 10 vol of cold acetone. The combined acetone supernatants were concentrated to a small volume and diluted with acetone to yield a second crop (yield of combined crops, 36.5 mg).

This crude 16:0-dCL, containing unreactied DPPA, was purified by preparative TLC using Solvent A, eluted from the silica with chloroform/methanol/water (1:2:0.8, by vol) and isolated as the ammonium salt as described above. The yield of TLC-pure 16:0-dCL ammonium salt was 14 mg (0.01 mmol, 10% from DPPA); R_f 0.60 in Solvent A. Fatty acid analysis by GLC: 14:0, 1%; 16:0, 95.5%; 16:1, 1%; 18:0, 1.6%; 18:1, 0.9%. ^1H NMR ($CDCl_3$): 0.86 (*t*, terminal CH_3, 12H), 1.23 [acyl CH_2, C-4 to C-(ω - 1), 96H], 1.55 (acyl C-3, 8H), 1.77 (*s*, unidentified, 4H), 2.08 (broad single peak, HOH), 2.28 (*t*, acyl C-2, 8H), 3.67 (*s*, unidentified, 3H), 3.9 (*m*, CH_2-O-P, 8H), 4.13 (*t*, P-O-C-CH_2-C-O-P, 2H), 4.33 (*d*, CH_2-O-acyl, *sn*-1-glycerol, 4H), 5.18 (*t*, CH-O-acyl, *sn*-2-glycerol, 2H). Negative FAB-MS: 811 (unidentified, possibly [M - dipalmitoylglycerol (DPG) + OH + Na - H]$^-$ or [phosphatidylpropanediol phosphate (PPDP) + Na - H]$^-$); (base peak) 705 [M - H -

DPPA + H_2O]$^-$ or [phosphatidylpropanediol (PPD) − H]$^-$; 647 [M − PPD + OH]$^-$ or [DPPA − H]$^-$; Positive FAB-MS: 707 [M − DPPA + H_3O]$^+$; 551 [M − PPDP + H]$^+$ or [DPG − OH]$^+$; calcd. for 16:0-dCL (free acid), $C_{73}H_{142}O_{16}P_2$, M = 1336; PPDP, $C_{38}H_{76}O_{12}P_2$, M = 786; PPD, $C_{38}H_{75}O_9P$, M = 706; DPPA, $C_{35}H_{69}O_8P$, M = 648; DPG, $C_{35}H_{68}O_5$, M = 568. No parent ion peak for 16:0-dCL was detected by negative or positive FAB-MS.

The free acid form of 16:0-dCL was prepared by the acidified Bligh and Dyer (29) procedure (see Ref. 30). The sodium salt was prepared by titration of the free acid form of 16:0-dCL0 with 0.05N NaOH in methanol to pH 7.5, followed by precipitation from chloroform solution with acetone.

Synthesis of the unsaturated deoxy analogue of CL. A synthesis of the unsaturated deoxy analogue of CL was carried out as described above for 16:0-dCL, but starting with commercial phosphatidic acid (PA) (0.08 mmole) derived from egg lecithin (Serdary, London, Ontario, Canada). The yield and TLC R_f values of the unsaturated dCL (yellow oil) were the same as for 16:0-dCL. Fatty acid composition: 14:0, 0.4%; 16:0, 39.2%; 16.1, 1.9%; 18:0, 10.9%; 18:1, 36.2%; 18.2, 9.8%; 18:3, 0.2%. ^{1}H NMR: 0.86 (*t*, terminal CH_3, 12 H), 1.23 (acyl-CH_2, C-4 to C-(ω − 1), 92 H), 1.56 (acyl C-3, 8 H), 1.75 (*s*, unidentified, 4 H), 2.00 (*q*, allylic methylene, 8 H), 2.28 (*q*, acyl C-2, 8 H), 2.76 (*t*, diallylic methylene, 2 H), 3.70 (*s*, unidentified, 4 H), 3.96 (*m*, CH_2-O-P, 8 H), 4.17 (*q*, PO-C-CH_2-C-OP, 2 H), 4.39 (*d*, CH_2-O-acyl, *sn*-1-glycerol, 4 H), 5.21 (*t*, H-C-O-acyl, *sn*-2-glycerol, 2 H), 5.32 (*m*, olefinic C-H, 5 H).

RESULTS

Syntheses. Because of the symmetry of the 2′-deoxy analogue of CL (16:0-dCL), its synthesis could be achieved by a single-step condensation of di-16:0-PA with 1,3-propanediol in the presence of pyridine or triethylamine and trichloroacetonitrile (Fig. 2). The 16:0-dCL was obtained in about a 10% overall yield but was contaminated with a black substance that co-precipitated with it and could only be removed by preparative TLC in Solvent A. The 16:0-dCL had a much higher mobility on TLC (R_f 0.60) in Solvent A than either natural CL or the hydrogenated CL (R_f 0.48), which can be attributed to the absence of the central OH group. Both the ^{1}H NMR and the FAB-MS spectra were consistent with the expected chemical structure of the dCL (Fig. 1). Under the conditions used here for FAB-MS, 16:0-dCL did not show a parent ion peak, the only negative ion peaks observed being those for the cleavage products, DPPA, PPD and possibly PPDP.

Potentiometric titration. H_2-CL-BH (18:0-CL) formed bilayer phases in either sonicated aqueous dispersion or in methanol/water (1:1, vol/vol) (21). On titration (Fig. 3), 18:0-CL showed the presence of one strong acid group, with apparent pK = 3.2, accounting for one equivalent of acid per mole, and a weak acid group with a surprisingly high pK accounting for a second equivalent of acid. The titration curve of the unhydrogenated BH-CL in methanol/water (1:1, vol/vol) was virtually superimposable on that of the 18:0-CL in water. Titration of the former in aqueous medium was not possible because this phospholipid forms hexagonal II phased dispersions in water (21), in which the polar head groups are not available for titration. In contrast, 16:0-dCL titrated as a normal dibasic acid in either sonicated aqueous dispersion (Fig. 3) or in methanol/water (1:1, vol/vol) (data not shown). It titrates two equivalents of base with an apparent pK of about 3.

Computer-simulated titration curves. An attempt was made to obtain more precisely the pK values of 18:0-CL (Fig. 3) by computer simulation assuming reasonable values for both the lower pK and the upper pK. It was apparent that this was not possible since a single value for pK_2 could not be obtained. As is shown in Figure 4, the experimental data crossed through a family of theoretical curves corresponding to pK_2 values varying from 7.5 to 9.5. Furthermore these values fit the lower portion of the curve best when pK_1 was assumed to be 2.8. It can be seen that the experimental curve differs from the theoretical curves by a "flattening" that is expected for titrations at polyanionic surfaces, on the basis of the calculations made by Tanford and Kirkwood (35) from Gouy-Chapman considerations. The second pK of CL appears to follow the formula: pK_2 = 7.5 + (fractional equivalent/2), approximating a linear increase in its pK value between 7.5 and about 9. CL thus "buffers" the protons throughout this range, entrapping protons up to a pH of 9.0 or greater.

In contrast, the titration of 16:0-dCL (Fig. 3) can be computer-stimulated (Fig. 5) by a theoretical curve for a typical dibasic acid having two strongly acidic groups with a pK_1 of 1.8 and pK_2 of 4.0. The "flattening" of the experimental curve here can also be accounted for by the surface charge effects desribed by Tanford and Kirkwood (35). Unlike the CL titration which displays an actual shift in the pK_2, dCL exhibits only a slight flattening of its titration curve (Fig. 5). This shows that the Gouy-Chapman effects are insufficient to provoke the dramatic pK_2 shift observed with CL (Fig. 4). It should also be noted that the results of Few *et al.* (11) show an upward drift after the pK_1 not unlike the titration reported here. Few *et al.* (11) obtained BH mitochondrial CL from Pangborn (5). Since there is no mention of hydrogenation, we must assume the CL was polyunsaturated and hence, we cannot be certain of the state of the CL (H_{II}, bilayer, *etc.*). Nevertheless, the upward drift of the titration curve obtained by Few *et al.* (11) appears to be consistent with our results.

Two other "titrations" have been reported in the literature for cardiolipin. Seddon *et al.* (13) reported a pK below 2.8 based upon the pH dependence of phase changes. This measurement could not be expected to detect a second pK unless there was a discrete phase change associated with it. These investigators assumed at the outset that there was only one pK and that CL is dibasic (16). The other attempt to measure the pK of CL (12) was made by titration in ethanol solution using indicator dyes. In this case a single pK was reported to be below 4.0 although a precise number could not be assigned.

Finally, the glycerol-1,3-diphosphate and 1,3-propanediol-1,3-diphosphate, model compounds for the polar headgroups of 18:0-CL and 16:0-dCL, respectively, both titrated as tetrabasic acids having two strong acid groups with pK 2.25 or 2.35, respectively, and two weak acid groups with pK 6.3 or 7.1, respectively (data not shown). The free hydroxyl on the glycerol-1,3-diphosphate does not appear to alter significantly the titration characteristics of the

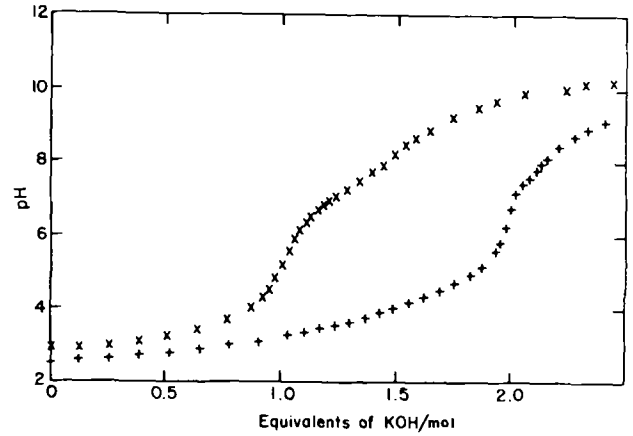

FIG. 3. pH-Titration curves of sonicated aqueous dispersions of hydrogenated beef heart cardiolipin (18:0-CL) (X) and of synthetic 2'-deoxycardiolipin (16:0-dCL) (+).

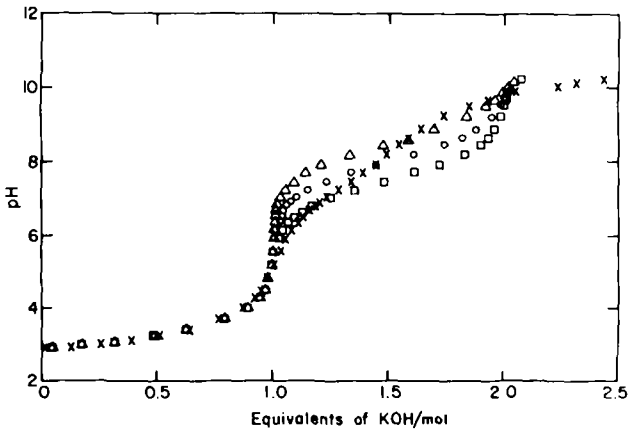

FIG. 4. Computer simulation of the titration of 18:0-CL: experimental curve (X); theoretical curves for a family of dibasic acids with pK_1 = 2.8, and pK_2 = 7.5 (□), 8.0 (△), 8.5 (○). Abbreviation as in Figure 3.

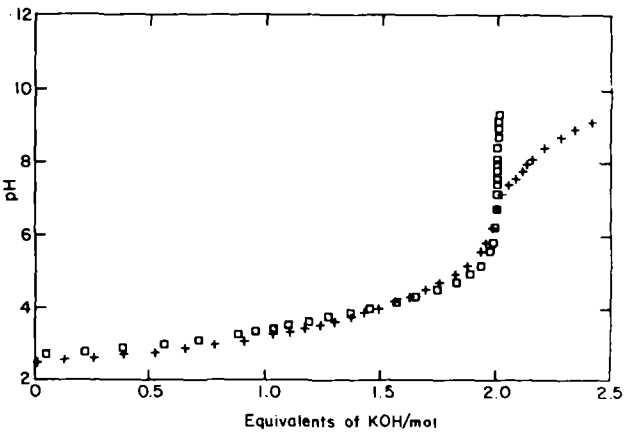

FIG. 5. Computer simulation of the titration of (16:0-dCL): experimental results (+); theoretical curve (□) for a dibasic acid with pK_1 1.8, pK_2 4.0. Abbreviation as in Figure 3.

phosphate groups since the propanediol-1,3-diphosphate displays essentially the same titration curve. However, see the discussion that follows.

DISCUSSION

The titration of the 18:0-CL presented in this paper is apparently the first electrometric titration of CL. Unlike previous attempts to titrate CL (11–13), the present measurements specifically examine the number of equivalents titrated at each pK. Our titration yielded two significant and surprising results. The first is that there are two pK values, the second pK (pK_2) being above physiological pH even at its lowest value. The second unexpected result is that the pK_2 itself changes rather dramatically as the titration proceeds. Furthermore, we have shown that in view of the comparison of the titration of CL to that of the dCL both of these unexpected results are due to the presence of the free hydroxyl on the connecting glycerol in CL.

The fact that dCL has two strong acid groups while CL has only one strong acid group may be attributed to the absence of the central OH in dCL, and to the presence of the central C-OH group in CL. The most likely way that this hydroxyl group can so significantly affect the pK of the phosphate groups is to form an intramolecular hydrogen bond with one of the P-OH groups (Fig. 6), analogous to the lipid acid anions described by Haines (36). The existence of such an intramolecular hydrogen bond has been demonstrated (37,38) by FTIR spectroscopy in phosphatidylglycerophosphate (now identified as phosphatidylglyceromethylphosphate, Ref. 39), as well as in CL (21).

The water-soluble headgroup moieties, glycerol-1,3-diphosphate and propanediol-1,3-diphosphate, do not display a significant difference in titration, as described in the Results section. This suggests that the difference between titrations of 18:0-CL and 16:0-dCL are due to the conformation of the headgroup of CL allowing the alignment of the C-OH and P-OH groups under constraint by the presence of the acyl groups (Fig. 6). It is this latter

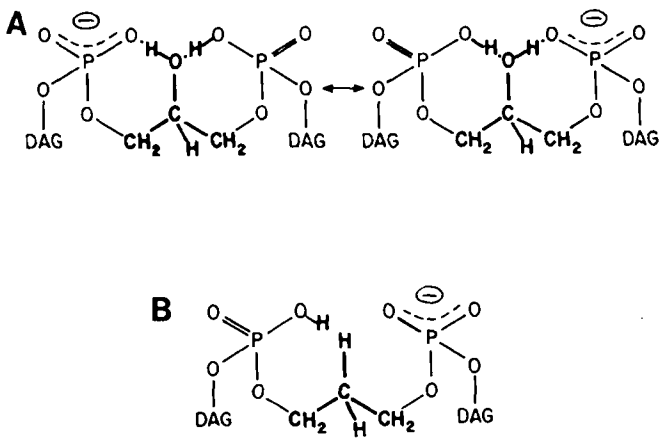

FIG. 6. A, proposed intramolecular hydrogen bonded acid-anion structure of cardiolipin at pH 7.5–9.5; and B, the structure of deoxycardiolipin at pH 2.0 (DAG, diacylglycerol). Note that structure A can trap a proton above pH 7.5 by forming an acid ion, while structure B cannot trap a proton, *i.e.*, cannot form an acid anion.

feature that also provides a mechanism to explain how the pK_2 of CL increases during the titration from 7.5 to 9.5 (Fig. 4).

The titration of dCL displays a pK_2 somewhat higher than its pK_1, presumably due to the suppression of ionization by the increased charge density on the surface as can be predicted from the work of Tanford and Kirkwood (35). In contrast, CL displays a shifting pK_2 as the titration proceeds. This suggests that the pK_2 of CL is sensitive to the charge density in two ways, first in the same way that dCL is, and second by a tightening of the H-bonded ring (Fig. 6). Presumably the second effect is significantly greater than the first; not only does a small increase in charge density provide a large increase in pK_2 but as the titration proceeds and the charge density increases, the pK shifts.

The biological significance of these observations pertains primarily to the capacity of CL to bind protons at relatively high pH. This might allow CL to buffer protons and provide a proton reservoir at high pH for participation in proton conduction in energy-transducing membranes as discussed by Haines and Kates (manuscript in preparation). These statements run counter to the suggestion that CL might provide protons at low pH (13).

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Modulating Effects of Bile Salt Hydrophobicity on Bile Secretion of the Major Protein of the Bile Lipoprotein Complex

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Bile lipids are secreted in association with a newly identified major apoprotein called anionic polypeptide fraction-calcium binding protein (APF-CBP), which is synthesized in the hepatocytes and has been detected in both bile and plasma and characterized. The secretion of the lipids in bile depends both on the concentration and the hydrophobicity of the bile salts (BS) secreted. The present study was undertaken to determine whether the synthesis and the secretion of APF-CBP are similarly regulated by BS, using two methods. The synthesis and secretion of labelled, newly synthesized APF-CBP by isolated rat hepatocytes were monitored by solid-phase immunoassay. For this purpose, hepatocytes were incubated with either glycodeoxycholate (GDC) or taurocholate (TC). The synthesis and secretion of labelled, newly synthesized APF-CBP by perfused rat liver were measured by immunological enzyme-linked assay (ELISA) upon perfusing the liver with either GDC or TC. We found that (i) the synthesis and the secretion of APF-CBP were increased during either TC or GDC perfusion, but the increase was more pronounced with TC; (ii) in GDC perfusion the APF-CBP levels measured were more closely related to the levels of bile salts and not to phospholipid levels, (iii) when the two bile salts were perfused in reverse order, *i.e.*, first GDC and then TC, the secretion of APF-CBP in bile decreased when GDC was perfused, but increased when TC was perfused. Similar results were obtained in experiments with isolated hepatocytes. The data suggest that the hydrophobicity of the BS used in the infusion modulates the synthesis and secretion of APF-CBP. In the liver, the pool of APF-CBP can be modified by BS and responds rapidly to BS stimulation.
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The relationship between biliary phospholipid (PL) secretion and bile acid levels suggests that the hydrophobic/hydrophilic balance of the bile salts (BS) (1) is an important aspect of bile regulation.

Until a decade ago, bile lipids were generally considered to be exclusively organized as mixed micelles (2). However, more recently, evidence of a far more complex organization has been emerging (3-5). Various structures have been implicated in the cholesterol transport in bile, including mixed micelles and large vesicles (3), as well as bile lipoprotein complexes (BLC) (4,5). BLC consist mainly of biliary lipids and a major apoprotein designated as anionic polypeptide fraction (APF).

APF has been isolated from human bile by zonal ultracentrifugation, and polyclonal and monoclonal antibodies have been obtained (6,7). APF has recently been identified as an apolipoprotein associated with bile lipids (5% of total PL weight) and serum high density lipoprotein (HDL_3) (0.6-1% of human apo HDL_3) (7). By weight, APF is the third most abundant protein in human (5) and rat bile (8). In cholesterol gallstones, APF has been detected at the pigment-cholesterol interface (6). Recently, another laboratory has independently isolated a 7 kDa amphipathic from bile (9) and various types of gallstones (9,10). It is a highly acidic polypeptide that contains covalently-bound bilirubin and shows a strong tendency to self-aggregate. The immunologic and functional identity of the polypeptide isolated from bile, APF, and gallstone calcium binding protein (CBP) has been established by collaboration between two laboratories (7) who have recommended the common designation APF-CBP. APF-CBP is the first bile-gallstone protein that has been shown to inhibit calcium carbonate precipitation and was also found to promote aggregation and fusion of biliary vesicles and crystallization of cholesterol therefrom (11).

APF-CBP was shown to be synthesized by both isolated and cultured rat hepatocytes (8) which have saturable and specific recognition sites for APF-CBP (12). APF-CBP has also been shown to be involved in the regulation of cholesterol uptake and cholesterol metabolism in the liver (13). In the rat, APF-CBP levels in bile are controlled by the nature of the bile acids (14), with cholic acid and dehydrocholic acid showing opposite effects. APF-CBP is associated with PL and cholesterol in bile, and the secretion of both these lipids depends on BS. Hence, it is important to determine whether APF-CBP secretion is directly modulated by BS and whether this modulation is a function of the hydrophobicity of the BS or whether it is under the control of other lipids, such as PL or cholesterol.

In the present study, we have compared the effects of two natural BS, namely taurocholate (TC) and glycodeoxycholate (GDC), on the synthesis and the secretion of APF-CBP in isolated perfused rat liver and in isolated hepatocytes. Using a relatively highly hydrophylic BS (TC) and a very hydrophobic BS (GDC) (15), we have also studied the effect of insulin, a hormone known to stimulate the synthesis of proteins in the liver (16).

MATERIALS AND METHODS

Liver perfusion. Male Sprague-Dawley rats weighing 250-300 g (IFFA Credo, l'Arbresle, France) with free access to food and water were used in all experiments. Animals were anesthetized by intraperitoneal sodium pentobarbital injection (60 mg/kg), and isolated liver was perfused in a single-pass system according to a slightly modified version of the technique described by Brauer *et al.* (17). First, the liver was exposed by abdominal incision and, after cannulation of the common bile duct, a 20-gauge intravenous Teflon catheter was immediately

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Abbreviations: ALT, alanine aminotransferase; APF-CBP, anionic polypeptide fraction-calcium binding protein; BLC, bile lipoprotein complex; BS, bile salts; GDC, glycodeoxycholate; HDL, high density lipoprotein; PL, phospholipid; TC, taurocholate.

placed in the portal vein, and perfusion was initiated at 37.0 ± 0.5°C. As soon as the liver turned white, the diaphragm was opened and the suprahepatic *vena cava* was cannulated with a 14-gauge Teflon catheter.

The perfusion medium was Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 2.5 mM $CaCl_2$) at pH 7.4 containing 10 mM glucose. Insulin at 5 U/100 mL (8), 0.6 mCi [^{14}C]leucine (specific activity 322 mCi/mmol or 11.8 GBq/mmol; CEA, Saclay, France) and 6 mL/100 mL RPMI 1640 (Flow Laboratories, Paris, France) were added to the medium. The complete medium was filtered through a 80-μm millipore filter using a peristaltic pump, and gassed to equilibrium with O_2/CO_2 (95:5, vol/vol). During perfusion, hydrostatic portal pressure was maintained constant at an average flow rate of 2.50 mL/min/g liver.

The experimental procedure involved a nonrecirculating system. After a 20-min equilibration period, during which the perfusion medium was infused at 12.5 nmol/min/g liver, the effect of the two BS upon bile lipid secretion was tested by infusing either 12.5 nmol/min/g liver TC or GDC over a 90-min period. When the rate of APF synthesis was assessed, [^{14}C]leucine (0.05 μmol/g liver) was infused with TC or GDC during the first 20 min.

In all experiments, bile was collected at 10-min intervals for 110 min in preweighed tubes. The perfusate was collected simultaneously from the supra-hepatic *vena cava*. Oxygen consumption by the liver was determined by measuring the oxygen saturation of the entering and exiting perfusate.

Preparation of isolated hepatocytes. The surgical procedure, as well as the purity and viability of the hepatocytes, were assessed as described previously (8). Hepatocytes were isolated according to the method of Berry and Friend (18) as modified by Seglen (19).

For incubations, hepatocytes 8–10 × 10^6 cells/mL were suspended in an incubation medium composed of fetal bovine serum (40 mL/L) supplemented with gentamycin (8 mg/L) and insulin (0.05 IU/mL). Ten-mL samples of medium were placed in 20-mL round glass flasks and incubated at 37°C under slow stirring with 95:5% O_2/CO_2. TC or GDC were added at concentrations of 0.2 μmol/mL and 0.17 μmol/mL, respectively.

Polyclonal (8) or monoclonal (7) antibodies against APF were prepared as previously described. APF synthesis was assessed by measuring the incorporation for 5 h of [^{14}C]-leucine, which was added to the incubation medium at a concentration of 5.4 μCi/mL. APF content was determined in supernatant samples by solid-phase radioimmunoassay (20) using a specific monoclonal mouse anti-human APF and a polyclonal rabbit anti-human APF reacting against rat APF (4). APF was bound to solid-phase CNBr-activated Sepharose 4B (Pharmacia, Saint Quentin, Yvelines, France) coupled with specific anti APF serum.

A-200 μL sample of supernatant from the incubation medium containing radiolabelled APF was mixed with a buffer composed of 0.05 M Na_2HPO_4, 0.35 M KCl, and 0.05 M DL-leucine at pH 7.2 to a final volume of 1.84 mL. A 100-μL sample was used with an excess of whole rat anti-serum (200 μL) in the same buffer to determine the nonspecific binding of radiolabelled leucine (21). A 200-μL sample of Sepharose coupled with specific antibodies was added to all the samples and incubated under continuous rotation for 3 h at room temperature. The resulting suspension was centrifuged at 200 × *g* for 5 min, and the pellet was washed three times in 5 mL of 0.05 M Na_2HPO_4 and 0.085 M KCl buffer (pH 7.2). The final pellet was suspended in 1 mL of a tissue solvent (Soluène 350; Packard, Rungis, France). After adding 0.1 mL of 30% H_2O_2 and incubating for 30 min at 50°C, the samples, upon cooling and solubilization, were counted after adding 10 mL of scintillation fluid (Instafluor; Packard). The radioactivity incorporated into APF was calculated for each sample by subtracting the nonspecific binding of other radioactive-labelled proteins as determined above.

Assays of bile and perfusate. BS concentrations were measured using the 3α-hydroxysteroid dehydrogenase assays (22). Radioactivity in bile and perfusate was measured by liquid scintillation counting as mentioned above. PL were determined by a semi-automatic colorimetric method (23). APF was detected and quantified by direct antigen binding enzyme-linked sandwich assay using polyclonal and monoclonal antibodies as described elsewhere (7).

Statistics. All data were statistically analyzed as the means ± SE, and the significance of the difference was determined using the unpaired Student's *t*-test. Probability values less than 0.05 were considered significant.

RESULTS

Bile flow. During the infusion with TC alone, the bile flow remained constant for 110 min. The bile flow of perfused rat liver stabilized at 18.8 ± 1.9 μL/min during TC infusion then decreased from 13.8 ± 1.3 μL/min to 9.5 ± 0.9 μL/min during GDC perfusion (Table 1). There was no difference between the two BS with regard to hepatic O_2 consumption, which was taken as a criterion of viability. Mean O_2 consumption was 2.5 ± 0.3 μmol/min/g liver for both groups, suggesting that no toxicity of BS was observed (24). Also, no elevation of bilirubin was noted. Alanine aminotransferase (ALT) elevation was observed with some BS, but no correlation was noted between

TABLE 1

Influence of Taurocholate (TC) Alone or TC Followed by Glycodeoxycholate (GDC) Infusion on Biliary Flow and Bile Acid Secretion[a]

	TC		TC/GDC	
Time (min)	Bile flow (μL/min)	Bile acid secretion (nmol/min)	Bile flow (μL/min)	Bile acid secretion (nmol/min)
10	18.5 ± 1.8	503 ± 80	18.8 ± 1.9	527 ± 68
20	18.6 ± 1.5	550 ± 70	18.6 ± 1.8	355 ± 50
30	17.2 ± 1.6	616 ± 51	13.8 ± 1.3	247 ± 58
40	17.0 ± 1.0	606 ± 58	11.0 ± 1.2	144 ± 46
50	16.8 ± 1.2	600 ± 41	11.3 ± 1.2	125 ± 63
60	16.2 ± 1.5	505 ± 27	12.0 ± 1.2	131 ± 72
70	19.2 ± 1.7	555 ± 41	10.4 ± 1.2	154 ± 74
80	19.4 ± 1.8	570 ± 29	10.4 ± 1.3	132 ± 83
90	18.2 ± 1.5	590 ± 33	9.9 ± 0.8	47 ± 11
100	20.2 ± 1.1	580 ± 29	10.3 ± 1.0	69 ± 19
110	19.9 ± 1.8	500 ± 31	9.5 ± 0.9	64 ± 13

[a]Data are means ± SE (n = 5). For the conditions of bile salt infusion, see the Materials and Methods section.

enzyme activity and hydrophobicity index. The only significant effect noted by us was a bile flow decrease.

Effects of TC and GDC on the secretion of lipids and APF-CBP into the bile of perfused rat liver. TC infused at 12.5 nmol/min/g liver induced physiological rates of lipid secretion (BS and PL) which remained stable throughout the experiment (BS range, 503-606 nmol/min; PL range, 71-79 nmol/min) (Fig. 1). The molar APF-CBP/BS and APF-CBP/PL ratios remained constant. The two curves (Fig. 1) showed a plateau at a mean of 1.7 for APF-CBP/BS and a mean of 12.7 for APF-CBP/PL.

GDC infused at 121.5 nmol/min/g liver induced a rapid decrease in PL secretion between 20 min and 110 min (Fig. 1). The secretion of APF-CBP also decreased, but not to the same extent as did PL. Thus, the molar APF-CBP/PL ratio varied from 25 to 5. By contrast, the decrease in APF-CBP and BS was the same, so the APF-CBP/BS molar ratio remained constant. During the experiment, the oxygen consumption did not change, and ALT activity remained in the physiological range of 3.2-23 IU/L.

Two procedures were used to study the effect of inverting the sequence of perfusion of BS on the APF-CBP secretion (Fig. 2): (i) TC (12.5 nmol/min/g liver) was infused for 30 min; then GDC (12.5 nmol/min/g liver) was infused for 60 min. The infusion of GDC after TC induced a dramatic decrease in APF-CBP secretion (Fig. 2A). (ii) GDC (12.5 nmol/min/g liver) was infused for 30 min and then TC for 60 min (Fig. 2B). The infusion of TC after GDC restored APF-CBP secretion in bile to near that observed with the TC perfusion (Fig. 2A).

Effect of TC and GDC on secretion of newly synthesized APF-CBP. The quantity of [^{14}C]leucine-labelled APF-CBP recovered from the bile of isolated perfused liver was the same after a 90-min perfusion with either TC or GDC, *i.e.*, 2.4 and 2.5 μg/whole liver (Fig. 3). However, the kinetics of the secretion changed. With TC infusion

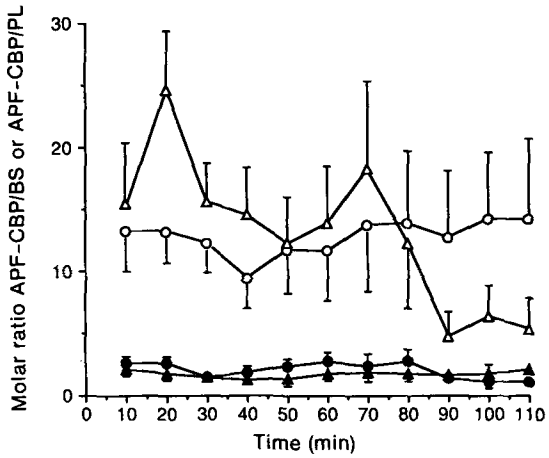

FIG. 1. Molar ratios of the secretion of bile lipids and anionic polypeptide fraction-calcium binding protein (APF-CBP) are shown as a function of time. Different components are expressed in nmol/min secreted from perfused rat liver during infusion of taurocholate (TC) or glycodeoxycholate (GDC) after a 20-min stabilization period with TC. Each point represents the mean ± SE (n = 5). The symbols used are APF-CBP/BS with TC (●), APF-CBP/PL with TC (○), APF-CBP/BS with GDC (▲), and APF-CBP/PL with GDC (△). BS, bile salts; PL, phospholipids.

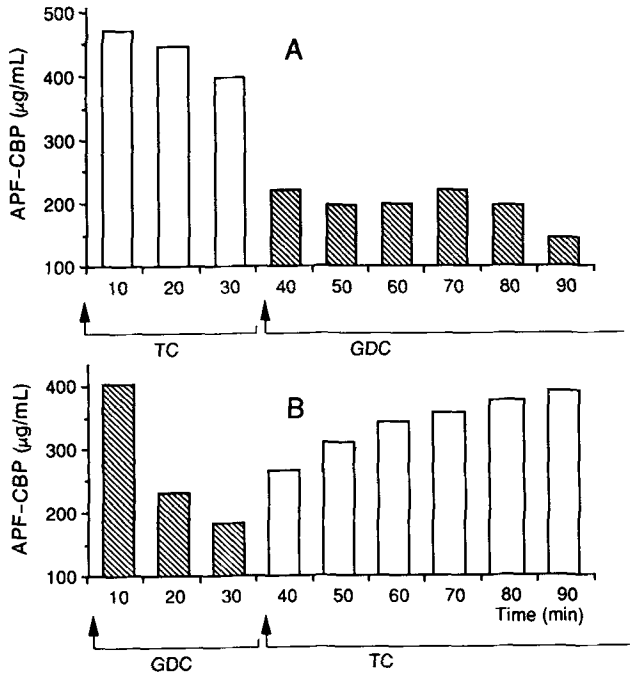

FIG. 2. Effect of inverting the sequence of perfusion of bile salts on the quantitative APF-CBP secretion in bile. A, APF-CBP secretion in bile from perfused rat liver after infusion of TC (30 min), followed by GDC (90 min) at a concentration of 12.5 nmol/min/g liver. B, APF-CBP secretion in bile from perfused rat liver after infusion of GDC (30 min) followed by TC (90 min) infusion. Output of APF-CBP is expressed in μg/mL (n = 2). The hatched bars represent the APF-CBP values with GDC infusion, the open bars after TC infusion. Abbreviations as in Figure 1.

(Fig. 3B), the maximum secretion of the labelled, newly synthesized protein was noted 40 min after perfusion of the labelled amino acid. With GDC infusion, the maximum secretion was noted 50 and 60 min after perfusion of amino acid (Fig. 3A). [^{14}C]Leucine in biliary APF-CBP represented 0.1% of the dose in the medium, and 1% of the [^{14}C]leucine was recovered in the liver.

No significant difference was noted in the effects of TC or GDC on APF-CBP synthesis by freshly isolated hepatocytes for 4 h (Table 2). With TC, the mean APF-CBP synthesis for this period was 0.819 μg/g of cells in the medium, and with GDC it was 0.693 μg/g cells. In both experiments, cell viability after 4 h of incubation was 85%. APF-CBP secretion was dependent on the addition of BS to the medium. The quantity of BS added to the medium was 0.20 μmol/mL for TC and 0.17 μmol/mL for GDC.

Effect of insulin on APF-CBP synthesis by isolated perfused liver. In the bile, the secretion of the labelled, newly synthesized APF-CBP in the presence of TC (12.5 nmol/min/g liver) is shown in Figure 3. When insulin was added at a dose of 5 IU/100 mL (Fig. 3B), the quantity of secreted protein doubled compared to the quantity obtained without insulin (Fig. 3C).

DISCUSSION

The results from the two experimental models used in this study demonstrate that APF-CBP secretion into bile is dependent on the presence of BS. APF-CBP secretion increased after either TC or GDC infusion, but GDC was

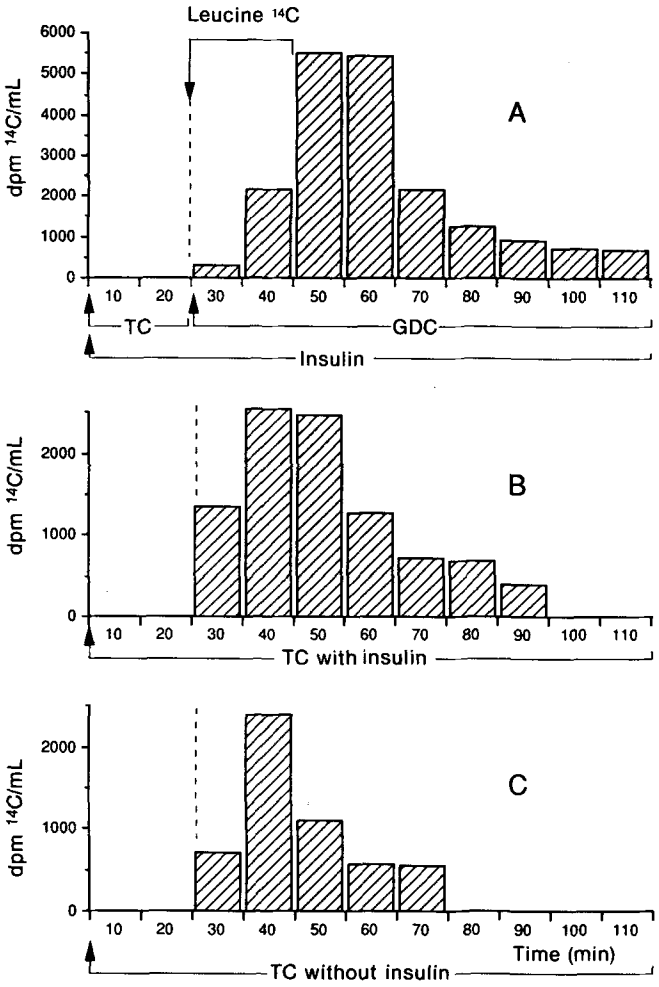

FIG. 3. Secretion of labelled APF-CBP by perfused rat liver. A, with GDC infusion at 12.5 nmol/min/g liver with insulin. B, with TC infusion at 12.5 nmol/min/g liver with insulin. C, with TC infusion at the same concentration without insulin. [^{14}C]leucine was infused for 20 min after TC equilibration. The values are expressed in dpm of [^{14}C]leucine quantified on APF-CBP secreted in bile (n = 3). Abbreviations as in Figure 1.

TABLE 2

Synthesis and Secretion of APF-CBP by Isolated Rat Hepatocytes Incubated with or without Bile Salts (BS)[a]

Time (h)	Without BS (mg/g cell)	With TC (mg/g cell)	With GDC (mg/g cell)
1	0.014 ± 0.020	0.147 ± 0.050	0.100 ± 0.020
2	0.053 ± 0.040	0.167 ± 0.080	0.157 ± 0.050
3	0.067 ± 0.055	0.205 ± 0.060	0.231 ± 0.040
4	0.087 ± 0.050	0.250 ± 0.050	0.205 ± 0.020
Total synthesis	0.221	0.819	0.693

[a]The cells were incubated in the presence of insulin and fetal calf serum. Samples were tested as described in the Materials and Methods section. Values are means ± SE of two determinations of anionic polypeptide fraction-calcium binding protein (APF-CBP) synthesized from cells prepared from four rat livers. APF-CBP determined in the supernatant is expressed in mg of APF-CBP/g of cells in the medium. The BS were added to the incubation medium at concentrations of 0.2 μmol/mL for TC and 0.17 μmol/mL for GDC. Abbreviations as in Table 1.

more effective in stimulating APF-CBP synthesis and secretion.

The results obtained on isolated perfused rat liver and isolated hepatocytes indicate that BS regulate the synthesis and secretion of APF-CBP. In contrast, insulin clearly enhanced only APF-CBP synthesis. The constant molar ratio of APF-CBP and BS furthermore emphasized the importance of BS in modulating this protein. Previous studies had focused on the binding of APF-CBP to biliary lipids. This association was especially strong with bile PL. For example, APF-CBP provoked a negative electrophoretic behavior of BLC depleted in BS and dissolved together with biliary lecithin in organic solvents (4).

Biliary secretion of PL and cholesterol depends strongly on the hydrophobicity of BS (24). Crawford *et al.* (25) have shown that bile flow, as well as PL and cholesterol levels in bile, were significantly correlated with TC secretion and that bile lipid secretion seemed to be related to the hydrophobicity of the BS.

Phosphatidylcholine in bile can originate from either the canalicular membranes (26), lysosomes (27) or microsomes (26) of liver. However, most likely, it originates from the microsomal membranes and our preliminary data indicate that APF-CBP most likely arises from microsomes as well (12). BS can have a detergent effect on microsomes, and several investigators have shown that the quantity of cholesterol and PL that was solubilized from these membranes increased with the hydrophobicity of the BS (15, 24); secretion of cholesterol and PL increased up to 50% depending on the degree of hydrophobicity of BS varying from 1.7 to 4.5 on the Bilhartz and Dietschy scale (1).

Although TC infusion induced a correlated, steady secretion of APF-CBP in bile, the levels of APF-CBP and PL in bile secretion varied independently when GDC was infused. Our data suggest that the BS could act as the initial modulator of APF-CBP secretion in bile.

The present study clearly showed that BS modulate the synthesis and secretion of APF-CBP and that, unlike other proteins, APF-CBP levels correlate with bile lipid secretion (28). Other studies in the rat had shown that albumin remained constant during BS infusion although APF-CBP was modulated by cholic acid and dehydrocholic acid (14). Furthermore, BS hydrophobicity was shown to determine how cholesterol is distributed between bile and plasma (15). A similar mechanism may also be operative with APF-CBP and could explain the distribution of APF-CBP in bile and plasma. The hydrophobicity of the BS may similarly affect phosphatidylcholine recruitment. When TC was infused after GDC, PL increased in bile suggesting that TC may mobilize a different pool of PL than the one mobilized by GDC.

The bipolar secretion of albumin has recently been demonstrated in hepatocytes (29); however, its BS dependence was not tested. The effect of BS upon APF-CBP secretion appears to be of considerable physiological relevance in regulating biliary secretion.

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On the Syntheses of Branched Saturated Fatty Acids

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In order to investigate the relation between the structure of mono-branched saturated fatty acids and their physical properties, a three-factor central composite design was constructed. For this purpose sixteen different fatty acids were prepared. The synthetic strategy was to use, if possible, a few common starting materials for the preparation of most of the acids. Thus alkylation and hydrolysis of oxazolines were used for the preparation of 2-butylhexanoic acid, 2-methyloctadecanoic acid, 2-hexadecyloctadecanoic acid and 2-pentyloctadecanoic acid. A number of acids were prepared from thiophene derivatives followed by desulfurization with Raney-Nickel alloy under alkaline conditions. Thus, starting from 3-ethylthiophene, 4-ethyl-2-thiophenecarboxylic acid and 4-ethyl-2-methyl-5-thiophenecarboxylic acid were prepared, which upon desulfurization gave the desired 4-methylhexanoic acid. From 3-bromo-2-methylthiophene, 3-ethyl-2-methyl-5-thiophenecarboxylic acid was prepared *via* 3-acetyl-2-methylthiophene and 3-ethyl-2-methylthiophene. Desulfurization gave 4-ethylhexanoic acid. Another approach started with 2-acylthiophenes, which were reacted with Grignard reagent to yield the appropriate olefins. By metalation and reaction with carbon dioxide, these were transformed to the corresponding 2-thiophenecarboxylic acids. Upon desulfurization, the desired fatty acids were obtained. In this way 6-propyldecanoic acid, 6-hexyldodecanoic acid, 6-methyldodecanoic acid and 6-pentylpentadecanoic acid were prepared. The remaining four acids were prepared from some of the branched acids described above through Kolbe reactions of dioic acids. Thus 16-methyloctadecanoic acid and 10-methyl-dodecanoic acid were obtained from 4-methylhexanoic acid, 16-ethyloctadecanoic acid from 4-ethylhexanoic acid and 9-pentyloctadecanoic acid from 6-pentylpentadecanoic acid.
***Lipids* 28, 889–897 (1993).**

Branched-chain fatty acids can be found in nature in many oils, fats and waxes, although in most cases the levels are very low. They also are minor components of many bacterial lipids, animal fats and marine oils (1). Bird waxes (from the preen gland) and lipids of gram-positive bacteria are important exceptions, where branched acids predominate (2,3). The preen gland wax of the Shanghai duck has a mono- and dimethyl-substituted fatty acid content of more than 85%. A total of 66 different methyl-branched saturated fatty acids have been identified from this species (4).

The industrial use of branched acids is very limited; only a few branched acids are produced today. The branching is almost exclusively either adjacent to the carboxyl group (α-branched) or near the end of the alkyl chain (*iso* or *anteiso* compounds). The α-branched compounds can be obtained by way of the Koch reaction, the oxo reaction or *via* aldol condensation followed by an oxidation of the aldehyde formed (5,6). Isostearic acid is a by-product in the dimerization process of unsaturated C_{18} fatty acids (7). 2-Ethyl-hexanoic acid, *iso*-stearic acid and the various *neo*-acids (formed in the Koch process from terminal olefins) are among the most important commercial branched-chain fatty acids (8,9).

The physical properties of branched-chain fatty acids are very different from straight-chain acids. The introduction of a branch alters many properties such as the melting point, the surface tension and the viscosity. The low pour point, low surface tension and the excellent oxidative stability of the acids and their derivatives make them useful in many applications. They have found use in cosmetics, lubricants, fabric softeners, etc. (5).

In order to investigate the relation between the structure of mono-branched saturated acids and their physical properties, a three-factor central composite design was constructed (10). The three factors involved are the length of the main chain, the length of the side chain and the branching position. This design required the preparation of fifteen different fatty acids. The results of this investigation are published in the following paper together with a more detailed discussion of the experimental design.

RESULTS AND DISCUSSION

According to the design discussed, the branched fatty acids 1–16 given in Table 1 were needed. Many of these acids have been described in the literature. However, we wanted to prepare these acids from as few starting materials as possible.

Therefore two main synthetic strategies were adopted. The α-branched acids were synthesized by alkylation of the lithium enolate of the dimethyl oxazolidines from the

TABLE 1

Branched Fatty Acids and References to Previous Methods for Their Preparation

Number	Name	Reference
1	2-Methylhexanoic acid	15-21
2	2-Butylhexanoic acid	18,22
3	4-Methylhexanoic acid	34,37
4	4-Ethylhexanoic acid	42
5	2-Methyloctadecanoic acid	23-26
6	2-Hexadecyloctadecanoic acid	27-29
7	16-Methyloctadecanoic acid	46,47
8	16-Ethyloctadecanoic acid	New
9	6-Propyldodecanoic acid	New
10	9-Pentyloctadecanoic acid	New
11	3-Ethylhexanoic acid	40,41
12	10-Methyldodecanoic acid	46,47
13	2-Pentyldodecanoic acid	31,32
14	6-Hexyldodecanoic acid	New
15	6-Methyldodecanoic acid	43-45
16	6-Pentylpentadecanoic acid	New

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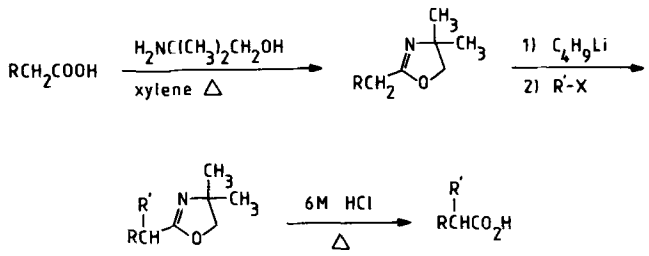
Abbreivations: BuLi, butyllithium; EI, electron impact; GC, gas chromatography; HPLC, high-performance liquid chromatography; IR, infrared; LDA, lithium diisopropylamide; MS, mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; TMEDA, tetramethylethanediamine.

proper unbranched acid (11), while the ω-2 and the internally substituted acids were synthesized *via* Raney-Nickel desulfurization of suitably substituted thiophenecarboxylic acids (12). In some cases the branched acids obtained upon desulfurization were chain-extended by use of an asymmetrical Kolbe synthesis (13,14).

Thus, **1** and **5** were prepared by alkylation of the enolate from 2-ethyl-4,5-dihydro-4,4-dimethyloxazole (propionic acid oxazoline) with butyl bromide and hexadecyl bromide, respectively (Scheme 1). The choice of propionic acid instead of hexanoic and octadecanoic acid as starting material for the oxazolidine synthesis could be motivated by the fact that both **1** and **5** might be synthesized from the same oxazoline.

However, a more important factor for the choice of the short acid is the simplicity gain in the purification of the branched oxazolines, especially in the case of **5** where the similarity of the product and the starting material would make the purification almost impossible. Similarly, heptanoic acid oxazoline and decyl bromide were used for the preparation of **13** and not dodecanoic acid oxazoline and pentyl bromide. Acid **2** was obtained *via* butylation of the hexanoic acid oxazoline, and **6** *via* octadecanoic acid oxazoline and hexadecyl bromide (Scheme 1).



R	R'	OXAZOLINE	ACID
CH_3	C_4H_9	21	1
C_4H_9	C_4H_9	22	2
CH_3	$C_{16}H_{33}$	23	5
$C_{16}H_{33}$	$C_{16}H_{33}$	24	6
C_5H_{11}	$C_{10}H_{21}$	25	13

SCHEME 1

Acid **1** is commercially available. There are also a number of methods for the preparation of **1**. Dialkylation of malonic ester followed by decarboxylation has been used (15). Acid **1** has been prepared by treating an oxazoline with lithium diisopropylamide (LDA) and alkylating the anion formed (16). Hexanol treated with a nickel catalyst and carbon monoxide gives **1** (17). Alkylation of the dianion of propionic acid also gives **1** (18). A common route is to add carbon monoxide to 1-hexene in the presence of a catalyst (19–21).

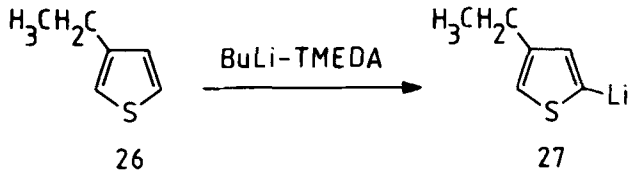
Acid **2** has been synthesized by treating the dianion of hexanoic acid with butyl bromide (18). It has also been prepared by decarboxylation of dibutylated malonic acid (22).

Several methods are available for the preparation of acid **5**. It has been prepared by reacting α-bromooctadecanoic acid with methylmagnesium iodide (23), and it has also been synthesized by a Kolbe electrolysis of hexadecanoic acid and 3-cyanobutanoic acid, followed by hydrolysis (24). A third alternative has been reported by Reppe and Kröper (25). They reacted octadecene with carbon monoxide in water in the presence of nickel carbonyl. Acid **5** has also been prepared by a dialkylation of diethyl malonate, followed by decarboxylation (26).

Acid **6** has been prepared by a malonic ester synthesis (27,28). It has also been synthesized from 1-hexadecene and octadecanoic acid in the presence of *t*-butyl peroxide (29). The method which we have adopted, converting the acid to its oxazoline and treating this with butyllithium followed by hexadecyl bromide, has been used before (30).

Acid **13** has been prepared by dialkylation of ethyl malonate followed by decarboxylation (31), and it was also synthesized from dodecanoic acid upon heating with *t*-butyl peroxide in the presence of 1-pentene (32).

It is well known that substituents can be selectively introduced in the thiophene ring when proper reaction conditions and reagents are used (33). This in combination with Raney-Nickel desulfurization, when the sulfur and the double bonds are removed, gives a saturated carbon skeleton. In organic synthesis, thiophene can therefore be used as a synthon for both straight and branched 4-carbon chains. Both of these possibilities have been exploited in this work.



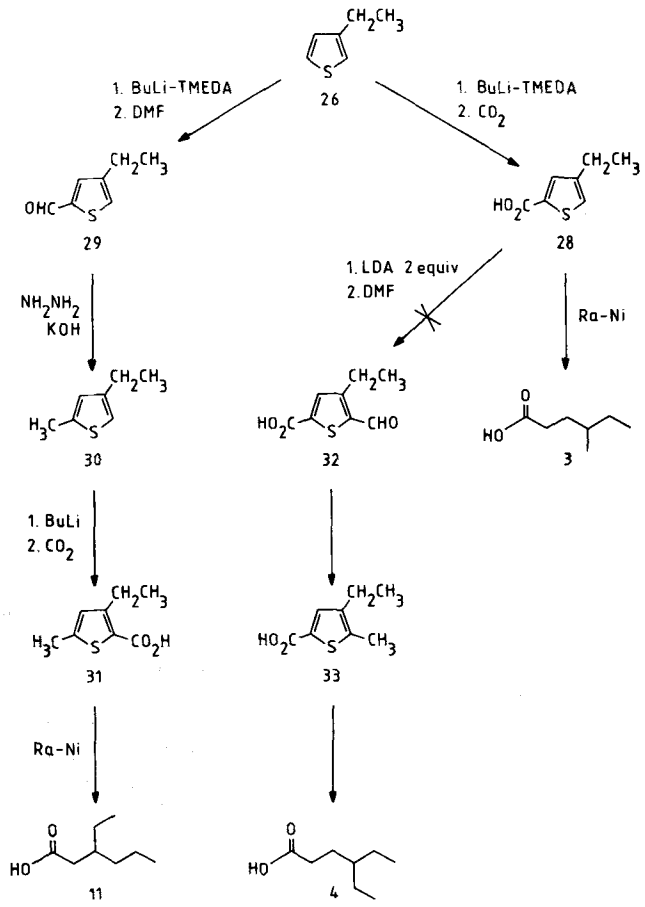
26 27

SCHEME 2

Our approach for the syntheses of the acid **11** and the (ω-2)-branched fatty acids was to use 3-ethylthiophene (**26**) as a key substance. If **26** is treated with one equivalent of the butyllithium-*N,N,N,N*-tetramethylethylenediamine (TMEDA) complex at room temperature, 4-ethyl-2-thienyllithium (**27**) will selectively be formed (Scheme 2) (33).

In the synthesis of 4-methylhexanoic acid (**3**), the lithiated 3-ethylthiophene (**27**) was treated with carbon dioxide to give 4-ethyl-2-thiophenecarboxylic acid (**28**) in 69% yield, which, upon Raney-Nickel desulfurization, gave **3** in 71–89% yield (Scheme 3).

Acid **3** has previously been synthesized in a number of ways. Wolff-Kishner reduction of the unsaturated aldehyde, citronellal yields 2,6-dimethyl-2-octene, which can be oxidatively cleaved by potassium permanganate (34) or ozone (35) to give **3**. This compound was also prepared from 1-bromo-2-methylbutane, formed from the corresponding alcohol, by treatment with diethyl malonate in the presence of sodium ethoxide, followed by decarboxylation (36). A third option is to use the reaction of succinic acid anhydride with ethyl- and methylaluminium chloride reagents to form a tertiary lactone, which can be



SCHEME 3

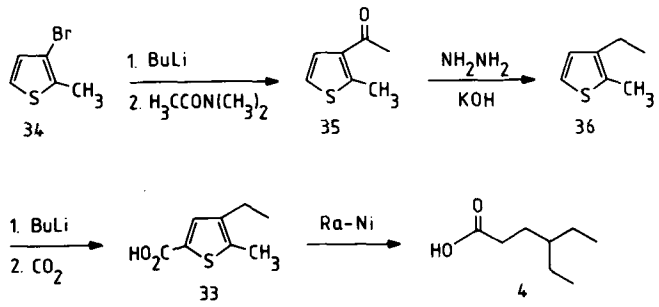
reductively opened by treatment with an organoaluminum halide reagent (37–39).

3-Ethylhexanoic acid (11) was also synthesized from 26, the lithiated derivative 27 reacted with *N,N*-dimethylformamide to give 4-ethyl-2-thiophene aldehyde (29), which was reduced to 4-ethyl-2-methylthiophene (30). Metalation of 30 with butyllithium followed by reaction with carbon dioxide gave 3-ethyl-5-methyl-2-thiophenecarboxylic acid (31) in 67–76% yield. After Raney-Nickel desulfurization, 11 was obtained in 73% yield (Scheme 3).

Acid 11 has previously been synthesized by reacting 3-bromohexane, synthesized from the corresponding alcohol, with either diethyl malonate (40), or acetic acid oxazoline (41).

Attempts to use 26 for the synthesis of 4-ethylhexanoic acid (4) *via* desulfurization of 3-ethyl-2-methyl-5-thiophenecarboxylic acid (33) were not successful. A direct lithiation of 28 with two equivalents of LDA followed by reaction with *N,N*-dimethylformamide failed to give 3-ethyl-2-formyl-5-thiophenecarboxylic acid (32), which we planned to reduce to 33. We then tried to prepare 2-iodo-4-ethylthiophene *via* metalation of 3-ethylthiophene followed by addition of iodine. The purpose of this approach was to use the iodine substituent as a protecting group, and then to be able to selectively introduce a formyl group to the 5-position by lithiation of the 2-iodo-4-ethylthiophene using one equivalent of LDA, followed by the addition of *N,N*-dimethylformamide. However, 2-iodo-4-ethylthiophene was formed in a mixture with 3-ethyl-2-iodothiophene.

We therefore prepared 33 from 3-bromo-2-methylthiophene (34) by converting it *via* halogen-metal exchange and reaction with *N,N*-dimethylacetamide to 3-acetyl-2-methylthiophene (35), which through Wolff-Kishner reduction was transformed to 3-ethyl-2-methylthiophene (36). Metalation followed by reaction with carbon dioxide gave 33, which upon Raney-Nickel desulfurization formed 4 in 77% yield (Scheme 4).

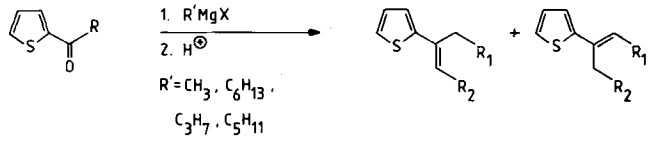


SCHEME 4

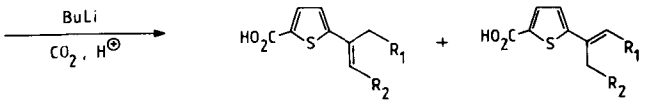
Acid 4 has previously been synthesized by reacting 2-ethyl butyraldehyde with malonic acid. The unsaturated acid was then hydrogenated using platinum oxide as catalyst (42). It has also been synthesized from succinic acid anhydride following the procedure described for acid 3 (37–39).

Another very useful starting material for several of the desired acids was 2-heptanoylthiophene (37) prepared by Friedel-Crafts acylation of thiophene with heptanoyl chloride using tin tetrachloride as catalyst (*cf.* Scheme 5). Reaction of this ketone with propyl magnesium bromide followed by dehydration gave a mixture of the isomeric olefins 38 and 39 in 73% yield. The mixture was metalated with butyllithium and reacted with carbon dioxide to give a mixture of the acids 40 and 41 in 78% yield, which was desulfurized to give 6-propyldodecanoic acid (9). The crude product obtained in 79% yield contained small amounts of unsaturated product, and was therefore hydrogenated over Pd on carbon using ethanol as solvent. As partial esterification occurred during the hydrogenation, the product was completely esterified, distilled and then hydrolyzed with sodium hydroxide to give pure 9 in an overall yield of 50%.

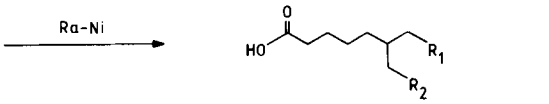
The acids 6-hexyldodecanoic acid (14) and 6-methyldodecanoic acid (15) were prepared in a similar way by treating the 2-heptanoylthiophene (37) with hexylmagnesium bromide and methylmagnesium iodide, respectively. The reaction of 2-heptanoylthiophene with methylmagnesium iodide gave, as in the first case, a mixture of the two olefines 44 and 45 in 64% yield. The isomeric distribution of this mixture could be determined by ^1H nuclear magnetic resonance spectroscopy (NMR). It was found that the least substituted olefin dominated, contrary to what might be expected. The overall yields of 6-hexyldodecanoic acid (14) and 6-methyldodecanoic acid (15) from heptanoylthiophene were 59 and 78%, respectively.



(37) R = C_6H_{13}	$R_1 = C_5H_{11}$, $R_2 = C_2H_5$	38	39
(48) R = C_9H_{19}	$R_1 = R_2 = C_5H_{11}$	42	
	$R_1 = C_5H_{11}$, $R_2 = H$	44	45
	$R_1 = C_8H_{17}$, $R_2 = C_4H_9$	49	50

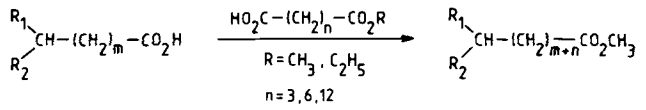


$R_1 = C_5H_{11}$, $R_2 = C_2H_5$	40	41
$R_1 = R_2 = C_5H_{11}$	43	
$R_1 = C_5H_{11}$, $R_2 = H$	46	47
$R_1 = C_8H_{17}$, $R_2 = C_4H_9$	51	52



$R_1 = C_5H_{11}$, $R_2 = C_2H_5$	9
$R_1 = R_2 = C_5H_{11}$	14
$R_1 = C_5H_{11}$, $R_2 = H$	15
$R_1 = C_8H_{17}$, $R_2 = C_4H_9$	16

SCHEME 5



Acid	R_1	R_2	m	Acid	R_1	R_2	m + n
3	C_2H_5	CH_3	2	7	C_2H_5	CH_3	14
4	C_2H_5	C_2H_5	2	8	C_2H_5	C_2H_5	14
16	C_9H_{19}	C_5H_{11}	4	10	C_9H_{19}	C_5H_{11}	7
				12	C_2H_5	CH_3	8

SCHEME 6

Acid 15 has been synthesized by Weitzel and Wojahn (43) using the same strategy as they used in the synthesis of 12, *i.e.*, repeated malonic ester syntheses. An alternative treatment of 4-methyl-1-decene with diethyl malonate in the presence of *t*-butyl peroxide gave 4-methyldecyl malonate, which upon decarboxylation yielded 15 (44). A third approach to 15 has been published by Asano *et al.* (45) in which hexyl zinc iodide and adipic acid chloride monoethyl ester were reacted to form ethyl 6-keto dodecanoate. The barium salt of the corresponding acid was treated with methylmagnesium iodide, and the product was dehydrated and reduced to give 15.

In order to obtain 6-pentylpentadecanoic acid (16), which was needed for the prepration of 9-pentyloctadecanoic acid (10), thiophene was acylated with decanoyl chloride to give 2-decanoylthiophene (48), which upon reaction with pentyl-magnesium bromide and dehydration in the same way as described above gave a mixture of the olefinic thiophenes 49 and 50. After metalation, treatment with carbon dioxide and desulfurization, 16 was obtained in 32% yield.

The remaining acids, 7, 8, 10, 12, were prepared by Kolbe syntheses (Scheme 6). The acids 7 and 12 were prepared by coupling of 3 with the monomethyl esters of tetradecanedioic acid and octadecanedioic acid, and the acid 8 by coupling of 4 with tetradecanedioic acid monomethyl ester. Finally acid 10 was obtained from 16 and the monomethyl ester of glutaric acid. Due to the formation of symmetrical coupling products, the yields obtained were only about 30%.

Acid 7 has been prepared in a multistep synthesis starting from 1-bromo-2-methylbutane. By extending the chain by repeatedly reacting the organocadmium compounds, synthesized from the corresponding halide, with mono acid chlorides to form keto esters, which could be reduced and converted to new saturated halides, which finally gave 7 (46). Milburn and Truter (47) have, in an approach similar to ours, used Kolbe syntheses for the preparation of 7. However, in their case 4-methyl hexanoic acid was coupled with two diacids in two consecutive steps to give 7.

Milburn and Truter (47) have also synthesized 12 by a Kolbe synthesis from 3-methylpentanoic acid and methyl hydrogen azelate. The 3-methylpentanoic acid was synthesized in three steps from 2-methyl-1-butanol. In another route, 12 has been prepared by repeated malonic ester synthesis, where the acids formed were reduced and halogenated and used again in new malonic ester syntheses (43).

MATERIALS AND METHODS

^{1}H NMR spectra were recorded on a Varian XL-300 spectrometer (Palo Alto, CA). The infrared (IR) spectrometer used was a Perkin-Elmer Model 298 (Norwalk, CT). Mass spectra were recorded on a Finnigan 4021 (San Jose, CA) and a Jeol JMS-SX 102 spectrometer (Tokyo, Japan). Gas chromatograms were obtained using Varian gas chromatographs models 1400 and 3300. Glass columns (length 2.0 m, i.d. 2 mm) were used with 10% SP-2340 and 3% OV 101 on Chromosorb Q 100/120. Careful analyses of the acids showed that they were of high purity (>98%).

2-Methylhexanoic acid (1) was prepared as described by Herslöf and Gronowitz (30). A 250-mL round-bottom flask equipped with reflux condenser was charged with 2-methylhexanoic acid oxazoline (10.0 g, 55 mmol) and 6M hydrochloric acid (55 mL). The reaction mixture was heated to reflux and kept at this temperature for 24 h. A hydrolyzed sample from the reaction mixture was analyzed by gas chromatography (GC), which showed no starting material. The reaction mixture was cooled and then poured into dichloromethane (50 mL). The organic phase was separated and the water phase was extracted twice with dichloromethane. The combined organic phases

were washed three times with 2M sodium hydroxide solution, after which the alkaline solution was acidified with concentrated hydrochloric acid under cooling. The product was dissolved in dichloromethane, and this solution was washed with water and dried over magnesium sulfate. Upon removal of the solvent, compound 1 (6.3 g, 88%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.9 (*t*, 3H, CH_3), 1.2 (*d*, 3H, CH_3), 1.25–1.35 (*m*, 4H, CH_2), 1.35–1.50 (*m*, 1H, CHH), 1.65–1.75 (*m*, 1H, CHH), 2.45 (*sext.* 1H, CHCO). Mass spectroscopy (MS) [electron impact (EI)] *m/e* (rel. int.): 115(0.7), 101(3), 87(21), 74(100). Known compound (15–21).

2-Butylhexanoic acid (2) was prepared as described above from 2-butylhexanoic acid oxazoline (10.0 g, 44 mmol) and 6M hydrochloric acid (44 mL). Evaporation of the solvent gave compound 2 (6.7 g, 88%). ^{1}H NMR ($CDCl_3$) δ 0.9 (*t*, 6H, CH_3), 1.25–1.40 (*m*, 8H, CH_3), 1.40–1.55 (*m*, 2H, CHH), 1.55–1.70 (*m*, 2H, CHH), 2.30–2.40 (*m*, 1H, CHCO). MS (EI) *m/e* (rel. int.): 173(1), 155(0.4), 143(2), 129(7), 116(45), 87(48), 73(100). Known compound (18,22).

4-Methylhexanoic acid (3). A 5-L three-necked round-bottom flask was equipped with a mechanical air-driven stirrer, the side-necks were equipped with two-necked adapters on one side for nitrogen and a funnel with a cork. The cork could be removed by pulling an iron wire attached to the cork. The two-necked adapter on the other side was equipped with a thermometer and two consecutive condensers. After sweeping the flask for 15 min with a strong stream of nitrogen, the flow was reduced and the flask was charged with 10% sodium hydroxide solution (2300 mL). During stirring, 4-ethyl-2-thiophenecarboxylic acid (28) (48) (30.0 g, 0.19 mol) was added. When 28 was dissolved, the solution was heated to 90°C, whereupon Raney-Nickel (240 g) was slowly added by means of a spoon through the funnel. It was important to add the alloy at a very moderate rate to avoid a too vigorous reaction. The temperature of the reaction mixture was kept at 90–100°C. When addition was completed, the reaction mixture was stirred at 90°C overnight. The hot reaction mixture was filtered through a glass filter, and the Raney-Nickel was washed twice with 150 mL of water. *It is important that the Raney-Nickel be kept wet, since it is pyrophoric.* The Raney-Nickel residue was then transferred to a glass bottle and covered with water. The combined alkaline water phases were cooled in an ice-bath, after which they were poured into ice-cooled concentrated hydrochloric acid (1500 mL). The acidic water solution was divided into two parts, and each part was extracted with three 500-mL portions of diethyl ether. The combined ether phases were washed with water and dried over magnesium sulfate. After evaporation of the solvent and distillation (b.p. 109–116°C/15 mm Hg), compound 3 (14.7 g, 89%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 0.89 (*d*, 3H, CH_3), 1.10–1.25 (*m*, 1H, CHH), 1.30–1.55 (*m*, 3H, CHH, CHH), 1.65–1.75 (*m*, 1H, CHH), 2.25–2.45 (*m*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 131(3), 113(2), 101(10), 83(15), 73(46), 71(76), 55(82), 41(100). Known compound (34,37).

4-Ethylhexanoic acid (4) was prepared analogously to 3 from 4-ethyl-5-methyl-2-thiophenecarboxylic acid (33) (25.0 g, 0.15 mol), Raney-Nickel (250 g) and 10% sodium hydroxide solution (2400 mL). After distillation (b.p. 126–131°C/15 mm Hg), compound 4 (16.4 g, 77%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.85 (*t*, 6H, CH_3), 1.20–1.35 (*m*, 5H, CH, CH_2), 1.55–1.65 (*m*, 2H, CH_2), 2.30 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 145(1), 115(6), 97(12), 85(42), 73(51), 69(47), 60(22), 55(49), 43(100). Known compound (42).

2-Methyloctadecanoic acid (5) was prepared analogously to 1 from 2-methyloctadecanoic acid oxazoline (10.0 g, 28 mmol) and 6M hydrochloric acid (29 mL). As 5 is a solid compound, the reaction mixture was transferred to a beaker and cooled in an ice-bath, whereupon the product separated as a solid phase, which was collected on a glass filter and washed twice with water. After recrystallization from ethanol (m.p. 54.5–56.0°C), pure 5 (5.7 g, 68%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3), 1.18 (*d*, 3H, CH_3), 1.20–1.35 (*m*, 28H, CH_2), 1.35–1.50 (*m*, 1H, CHH), 1.60–1.75 (*m*, 1H, CHH), 2.40–2.50 (*m*, 1H, CHCO). MS (EI) *m/e* (rel. int.): 298(5), 255(3), 241(4), 227(2), 199(4), 185(4), 143(7), 129(7), 87(33), 74(86), 43(100). Known compound (23–26).

2-Hexadecyloctadecanoic acid (6) was prepared analogously to 5 from 2-hexadecyloctadecanoic acid oxazoline (30) (10.0 g, 18 mmol) and 6M hydrochloric acid (17 mL). After recrystallization from ethanol (m.p. 74.0–76.5°C), compound 6 (7.5 g, 82%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.15–1.35 (*m*, 56H, CH_2), 1.40–1.55 (*m*, 2H, CHH), 1.55–1m70 (*m*, 2H, CHH), 2.30–2.40 (*m*, 1H, CHCO). MS (EI) *m/e* (rel. int.): 508(6), 409(1), 395(1), 381(1), 297(7), 284(20), 241(6), 185(8), 129(16), 57(98), 43(100). Known compound (27–29).

16-Methyloctadecanoic acid (7). A 500-mL flask, equipped with a thermometer and a magnetic stirring bar, was charged with 4-methylhexanoic acid (3) (5.0 g, 38 mmol), monomethyl ester of tetradecanedioic acid (21.0 g, 77 mmol) and potassium hydroxide (0.6 g, 11.5 mmol) in methanol (400 mL). A platinum electrode (12 cm^2, as anode) and an iron electrode (as cathode) were inserted into the solution, which was electrolyzed using a constant voltage. The initial current density was 175 mA/cm^2, and the temperature of the reaction mixture was kept at 40–55°C. The volume of the reaction mixture was kept constant by adding methanol during the reaction. When 1.1 F/mol had passed through the cell, the reaction was stopped. The reaction mixture was treated with a mixture of diethyl ether, dichloromethane and 1M hydrochloric acid. The phases were separated and the organic phase washed with sodium carbonate solution and water, dried over magnesium sulfate and evaporated. The residue was distilled (b.p. 136–142°C/0.05 mm Hg), and methyl ester of 16-methyloctadecanoic acid (3.0 g, 26%) was obtained.

In order to hydrolyze the ester, a mixture of ester (3.0 g, 10 mmol) and sodium hydroxide (3.0 g, 77 mmol) in ethanol (2 mL) and water (20 mL) was refluxed for 5 h. The reaction mixture was poured into a beaker and cooled to room temperature. The precipitate formed was treated with concentrated hydrochloric acid (7.5 mL) and diethyl ether/dichloromethane (1:1, vol/vol). The phases were separated and the organic phase washed with water, dried over magnesium sulfate and evaporated, giving pure 7 (2.9 g, 93%) with m.p. 49.0–50.5°C. ^{1}H NMR ($CDCl_3$) δ 0.85 (*t*, 3H, CH_3), 0.84 (*d*, 3H, CH_3), 1.05–1.20 (*m*, 1H, CHH), 1.20–1.40 (*m*, 26H, CHH, CH, CH_2), 1.58–1.70 (*m*, 2H, CH_2), 2.35 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 298(6), 281(2), 269(3), 251(4), 199(5), 185(5), 129(10), 71(39), 43(100). Known compound (46,47).

16-Ethyloctadecanoic acid (8) was prepared analogously to 7 from 4-ethylhexanoic acid (4) (5.0 g, 35 mmol), monomethyl ester of tetradecanedioic acid (19 g, 70 mmol) and potassium hydroxide (0.6 g, 11.5 mmol) in methanol (400 mL). Distillation (b.p. 187–189°C/2 mm Hg) gave the ester (3.5 g, 31%), which upon hydrolysis gave 8 (3.2 g, 96%) with m.p. 51.0–54.0°C. ^{1}H NMR ($CDCl_3$) δ 0.78 (*t*, 6H, CH_3), 1.10–1.35 (*m*, 29H, CH_2, CH), 1.50–1.65 (*m*, 2H, CH_2), 2.20 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 312(6), 294(3), 283(6), 256(6), 242(4), 111(9), 98(13), 84(26), 71(31), 55(55), 43(100). Calc. for $C_{20}H_{40}O_2$: C, 76.86; H, 12.90; MW 312.54. Found: C, 74.89; H, 12.60.

6-Propyldodecanoic acid (9). The first attempt was analogous to the preparation of 3 from a 10% sodium hydroxide solution (2500 mL), ethanol (1000 mL), a mixture of 4-(5′-carboxy-2′-thienyl)-3-decene (31) and 4-(5′-carboxy-2′-thienyl)-4-decene (32) and Raney-Nickel (200 g). GC analysis of the crude product showed that 38% of the starting material was left. The crude product was therefore desulfurized twice, each time using 10% sodium hydroxide solution (830 mL), ethanol (330 mL) and Raney-Nickel (67.0 g). After this procedure, GC analysis showed that the starting material was consumed. However, traces of unsaturated compounds were detected. The product (17.0 g) was therefore hydrogenated in ethanol (100 mL) with hydrochloric acid (3 drops) over palladium on charcoal (0.5 g) at 50°C and a hydrogen pressure of 50 psi. After 72 h, the reaction was interrupted and the catalyst was removed by filtration through celite. The filtrate was treated with concentrated hydrochloric acid (2 mL) and refluxed for 4 h. The reaction mixture was cooled, poured into brine (50 mL), and the product was taken up in diethyl ether (3 × 150 mL). The combined ether phases were dried over magnesium sulfate and evaporated, whereupon the residue was distilled (b.p. 103–108°C/0.25 mm Hg) giving ethyl 6-propyl dodecanoate (12.2 g, 60%).

The ester was mixed with water (65 mL), ethanol (13 mL) and sodium hydroxide (11.0 g, 0.26 mol), and the reaction mixture was refluxed for 5 h. After cooling and acidification with concentrated hydrochloric acid, the product was taken up in diethyl ether, and the ether solution was dried over magnesium sulfate. Evaporation gave pure 9 (10.2 g, 93%). ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 0.88 (*t*, 3H, CH_3), 1.15–1.35 (*m*, 19H, CH_2, CH), 1.55–1.70 (*m*, 2H, CH_2), 2.36 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.) $Si(CH_3)_3$ derivative: 314(4), 299(20), 285(2), 271(5), 299(5), 180(14), 145(36), 132(45), 117(70), 73(100). Calc. for $C_{15}H_{30}O_2$: C, 74.32; H, 12.47; MW 242.41. Found: C, 74.95; H, 12.56.

9-Pentyloctadecanoic acid (10) was prepared analogously to 7 from 6-pentylpentadecanoic acid (16), monomethyl ester of pentanedioic acid (37.4 g, 26 mmol) (49), potassium hydroxide (0.90 g, 16 mmol) and methanol (400 mL). After work-up and distillation (184–191°C/0.7 mm Hg), methyl 9-pentyloctadecanoate (5.9 g, 25%) was obtained. The ester was hydrolyzed using water (60 mL), ethanol (10 mL) and sodium hydroxide (5.0 g, 125 mmol). After work-up, compound 10 (5.0 g, 89%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (, 6H, CH_3), 1.20–1.40 (*m*, 35H, CH_2, CH), 1.57–1.70 (*m*, 2H, CH_2), 2.35 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 355(1), 283(2), 260(2), 227(4), 209(4), 111(8), 97(13), 85(18), 71(35), 57(75), 43(100). Calc. for $C_{23}H_{46}O_2$: C, 78.02; H, 13.00; MW 356.62. Found: C, 78.14; H, 13.19.

3-Ethylhexanoic acid (11) was prepared analogously to 3 from 10% sodium hydroxide solution (2400 mL), 3-ethyl-5-methyl-2-thiophenecarboxylic acid (31) (25.0 g, 0.15 mol), and Raney-Nickel (250 g). After work-up and distillation (b.p. 120°C/13 mm Hg) 11 (14.8 g, 73%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.20–1.50 (*m*, 6H, CH_2), 1.75–1.90 (*m*, 1H, CH), 2.28 (*d*, 2H, CH_2). MS (EI) *m/e* (rel. int.): 145(1), 115(5), 101(7), 84(30), 60(90), 43(100). Known compound (40,41).

10-Methyldodecanoic acid (12) was prepared analogously to 7 from 4-methylhexanoic acid (3) (10.0 g, 76.9 mmol), monoethyl ester of octanedioic acid (50) (31.1 g, 154 mmol), potassium hydroxide (1.3 g, 23 mmol) and methanol (450 mL). Work-up and distillation (b.p. 105–110°C/1 mm Hg) gave ethyl 10-methyl dodecanoate (7.6 g, 1 mm Hg). The ester was hydrolyzed using water (50 mL), ethanol (5 mL) and sodium hydroxide 3.8 g, 95 mmol). Work-up and evaporation gave 12 (6.0 g, 84%). ^{1}H NMR ($CDCl_3$) δ 0.88 (*d*, 3H, CH_3), 0.89 (*t*, 3H, CH_3), 1.05–1.20 (*m*, 1H, CHH), 1.20–1.45 (*m*, 14H, CH, CHH, CH_2), 1.55–1.70 (*m*, 2H, CH_2), 2.30 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.) ($Si(CH_3)_3$ derivative: 286(3), 271(16), 257(3), 243(3), 227(3), 201(5), 145(4), 132(22), 117(50), 75(70), 73(100). Known compound (46,47).

2-Pentyldodecanoic acid (13) was prepared analogously to compound 5 from 2-pentyldodecanoic acid oxazoline (105.3 g, 0.33 mol) and 6M hydrochloric acid (330 mL). After 96 h the reaction mixture was worked up and 13 (59.4 g, 67%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.20–1.40 (*m*, 24H, CH_2), 1.40–1.55 (*m*, 2H, CHH), 1.55–1.70 (*m*, 2H, CHH), 2.35 (*m*, 1H, $CHCO$). MS (EI) *m/e* (rel. int.): 270(4), 213(6), 143(15), 130(62), 87(71), 73(100), 43(90). Known compound (31,32).

6-Hexyldodecanoic acid (14) was prepared analogously to 9 from 10% sodium hydroxide solution (2460 mL), ethanol (990 mL), 7-(5′-carboxy-2′-thenyl)-6-tridecene (43) (20.0 g, 65 mmol) and Raney-Nickel (197 g). After work-up pure 14 (10.9 g, 59%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.15–1.35 (*m*, 25H, CH_2, CH), 1.55–1.70 (*m*, 2H, CH_2), 2.36 (*t*, 2H, CH_2). MS (EI) *m/e* (rel. int.): 285(4), 266(3)m, 248(5), 222(22), 199(9), 181(11), 163(10), 102(25), 57(79), 43(100). Calc. for $C_{18}H_{36}O_2$: C, 75.99; H, 12.76; MW, 284.49. Found: C, 76.19; H, 13.12.

6-Methyldodecanoic acid (15) was prepared analogously to 9 from 10% sodium hydroxide solution (2500 mL), ethanol (1000 mL), the mixture of 2-(5′-carboxy-2′-thienyl)-1-octen (46) and 2-(5′-carboxy-2′-thienyl)-2-octene (47) (20.0 g, 84 mmol) and Raney-Nickel (200 g). After work-up and distillation (b.p. 131–136°C/1.1 mm Hg) 15 (14.2 g, 78%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.84 (*d*, 3H, CH_3), 1.05–1.15 (*m*, 1H, CHH), 1.20–1.40 (*m*, 14H, CH_2, CH), 1.55–1.65 (*m*, 2H, CH_2), 2.36 (*t*, 2H, CH_2CO). MS (EI) *m/e* (rel. int.): 215(9), 197(7), 179(6), 152(29), 129(8), 111(7), 83(39), 55(65), 43(100). Known compound (43–45).

6-Pentylpentadecanoic acid (16) was prepared analogously to 9 from 10% sodium hydroxide solution (1500 mL), ethanol (1000 mL), the mixture of 6-(5′-carboxy-2′-thienyl)-6-pentadecene (51) and 6-(5′-carboxy-2′-thienyl)-6-pentadecene (52) (30.0 g, 89 mmol) and Raney-Nickel (120 g). The crude product (17 g) was hydrogenated and esterified giving upon distillation (b.p. 177–180°C/0.40 mm Hg) ethyl 6-pentylpentadecanoate (11.6 g, 99%). ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 6H, CH_3), 1.15–1.35 (*m*, 29H, CH_2, CH), 1.55–1.65 (*m*, 2H, CH_2), 2.36 (*t*, 2H, CH_2CO).

MS (EI) *m/e* (rel. int.) 312(0), 294(6), 276(10), 250(100), 241(13), 223(15), 185(20), 167(28). Calc. for $C_{20}H_{40}O_2$: C, 76.86; H, 12.90; MW, 312.54. Found: C, 76.64; H, 13.08.

Propionic acid oxazoline (**17**) (51) was prepared analogously to acetic acid oxazoline (52). A 500-mL round-bottom flask equipped with a thermometer, magnetic bar and reflux condenser was charged with freshly distilled 2-amino-2-methyl-1-propanol (178 g, 2.0 mol), and propionic acid was added. With stirring the reaction mixture was refluxed until the reflux temperature dropped from 165 to 142°C. The oxazoline was distilled azeotropically at 95–125°C through a Vigreux column. The distillate was collected in a receiver containing 300 mL of hexane, in which the water separated as a lower layer. The water layer was extracted three times with hexane, and the combined organic phases were dried over magnesium sulfate. After evaporation and distillation (b.p. 130–131°C) compound **17** (177.4 g, 70%) was obtained.

Hexanoic acid oxazoline (**18**) was prepared according to (30) from hexanoic acid (116 g, 1.0 mol) and 2-amino-2-methyl-1-propanol (116 g, 1.3 mol) in xylene (100 mL). The flask was equipped with a water estimator and the reaction mixture was refluxed for 2 h, whereupon the xylene was distilled off. The residue was chromatographed on neutral alumina (Merck, Darmstadt, Germany) using diethyl ether as eluent. After evaporation and distillation (b.p. 83–85°C/19 mm Hg) **18** (105 g, 62%) was obtained. Lit. value (30) b.p. 90–92°C/25 mm Hg.

Octadecanoic acid oxazoline (**19**) was prepared analogously to **18** from octadecanoic acid (284 g, 1.0 mol) and 2-amino-2-methyl-1-propanol (116 g, 1.3 mol). Distillation (138–143°C/0.025 mm Hg) gave compound **19** (275 g, 82%). Lit. value (30) b.p. 90–92°C/0.01 mm Hg.

Heptanoic acid oxazoline (**20**) was prepared analogously to **18** from heptanoic acid (130 g, 1.0 mol) and 2-amino-2-methyl-1-propanol (116 g, 1.3 mol). After reflux for 36 h at 180–190°C and work-up followed by distillation (b.p. 98°C/18 mm Hg), compound **20** (122.6 g, 67%) was obtained. Lit. value (53) b.p. 96–98°C/15 mm Hg.

2-Methylhexanoic acid oxazoline (**21**). A 500-mL round-bottom flask equipped with a nitrogen inlet, dropping funnel and magnetic bar was charged with propionic acid oxazoline (**17**) (10.0 g, 79 mmol) in tetrahydrofuran (200 mL) and cooled to −78°C. Butyllithium (58 mL, 1.50 M) in hexane was added dropwise, and the reaction mixture was stirred for 1.5 h. Butyl bromide (10 mL, 94 mmol) in tetrahydrofuran (120 mL) was then added. One hour later the cooling bath was removed, and the reaction mixture was allowed to reach room temperature overnight. The mixture was poured into saturated ammonium chloride solution, diethyl ether was added and the phases were separated. The water phase was extracted twice with ether, and the combined organic phases were washed with saturated sodium chloride solution and dried over magnesium sulfate. After evaporation and distillation (b.p. 110–114°C/53 mm Hg) compound **21** (11.0 g, 76%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.90 (*t*, 3H, terminal CH_3), 1.10 (*d*, 3H, terminal CH_3), 1.20–1.35 (*m*, 10H, CH_2, ring CH_3), 1.40–1.50 (*m*, 1H, CHH), 1.55–1.70 (*m*, 1H, CHH), 2.43 (*sext.*, 1H, CH), 3.90 (*s*, 2H, ring CH_2). MS (EI) *m/e* (rel. int.) 183(2), 168(13), 154(6), 140(34), 127(100). 112(18).

2-Butylhexanoic acid oxazoline (**22**) was prepared as described for **21** from hexanoic acid oxazoline (**18**) (10.0 g, 59 mmol) in tetrahydrofuran (150 mL), butyllithium (45 mL, 1.44 mol) in hexane and butyl bromide (7.7 mL, 71 mmol) in tetrahydrofuran (90 mL). After evaporation, the residue was flash-chromatographed on silica using hexane/diethyl ether (9:1, vol/vol) as eluent, and compound **22** (7.7 g, 58%) was obtained pure. ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 6H, terminal CH_3), 1.20–1.35 (*m*, 14H, CH_2, ring CH_3), 1.40–1.60 (*m*, 4H, CH_2), 2.30–2.40 (*m*, 1H, CH), 3.90 (*s*, 2H, ring CH_2). MS (EI) *m/e* (rel. int.) 225(0.4), 210(3), 196(1), 182(16), 169 (14), 140(24), 126(100).

2-Methyloctadecanoic acid oxazoline (**23**) was prepared as described for **21** from propionic acid oxazoline (**17**) (10.0 g, 79 mmol) in tetrahydrofuran (200 mL), butyllithium in hexane (58 mL, 1.50 M) and hexadecyl bromide (29.0 g, 94 mL) in tetrahydrofuran (300 mL). After work up and distillation (b.p. 136–144°C/0.03 mm Hg) compound **23** (17.0 g, 62%) was obtained. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3), 1.15 (*d*, 3H, CH_3), 1.20–1.35 (*m*, 36H, ring CH_3, CH_2), 2.37–2.50 (*sext.*, 1H, CH), 3.88 (*s*, 2H, ring CH_2). MS (EI) *m/e* (rel. int.) 351(3), 336(2), 322(1), 308(3), 294(2), 182(6), 140(31), 127(100).

2-Hexadecyloctadecanoic acid oxazoline (**24**) was prepared (30) from octadecanoic acid oxazoline (**19**) (10.0 g, 30 mmol) in tetrahydrofuran (350 mL), butyllithium in hexane (20 mL, 1.50 M) and hexadecyl bromide (11.0 g, 36 mmol) in tetrahydrofuran (120 mL). Work-up and distillation (b.p. 240–275°C/0.04 mm Hg) gave compound **24** (12.0 g, 70%). Lit. value (30) m.p. 76.5–78.0°C.

2-Pentyldodecanoic acid oxazoline (**25**) was prepared as described for **21** from heptanoic acid oxazoline (**20**) (120.0 g, 0.65 mol) in tetrahydrofuran (325 mL), butyllithium in hexane (473 mL, 1.51 M) and decyl bromide (161.0 g, 0.73 mol) in tetrahydrofuran (200 mL). Work-up and distillation (b.p. 128–142°C/0.05 mm Hg) gave compound **25** pure (105.0 g, 50%). ^{1}H NMR ($CDCl_3$) δ 0.83–0.90 (*dt*, 6H, termal CH_3), 1.20–1.35 (*m*, 28H, ring CH_3, CH_2), 1.38–1.61 (*m*, 4H, CH_2), 2.23–2.29 (*m*, 1H, CH), 3.90 (*s*, 2H, ring CH_2). *MS (EI) m/e* (rel. int.) 323(5), 308(4), 226(8), 196(18), 183(20), 126(100), 43(75).

3-Ethyl-5-methyl-2-thiophenecarboxylic acid (**31**). A 500-mL three-necked flask was equipped with a two-necked adapter, stirrer, condenser with drying tube, nitrogen inlet and dropping funnel. After flushing with nitrogen, the apparatus was charged with 2-methyl-4-ethylthiophene (33) (11.9 g, 94 mmol) in anhydrous diethyl ether (100 mL). Butyllithium in hexane (68.2 mL, 1.51 M), diluted with dry ether (100 mL), was added dropwise at room temperature. When the addition was complete, the reaction mixture was refluxed for 15 min, after which it was poured onto solid carbon dioxide covered with dry ether. At 0°C, water (200 mL) was added, the phases were separated and the ether phase was extracted twice with 2M sodium hydroxide solution. The combined aqueous phases were acidified with 1M sulfuric acid, and the precipitate was filtered off. Recrystallization from ethanol/water gave compound **31** (10.7 g, 67%) with m.p. 128.0–129.0°C. Calc. for $C_8H_{10}O_2S$: C, 56.46; H, 5.92; MW, 170.23. Found: C, 56.31; H, 5.91; MW, 170.

4-Ethyl-5-methyl-2-thiophenecarboxylic acid (**33**) was prepared analogously to **31** from 3-ethyl-2-methylthiophene (**36**) (33) (19.3 g, 153 mmol) in dry diethyl ether (100 mL) and butyllithium in hexane (112 mL, 1.50 M) diluted with dry ether (100 mL). Recrystallization from ethanol/water gave compound **33** (17.7 g, 68%) with m.p.

106–108°C. Calc. for $C_8H_{10}O_2S$: C, 56.46; H, 5.92; MW, 170.23. Found: C, 56.49; H, 5.94; MW, 170.

2-Heptanoylthiophene (**37**) was prepared according to Reference 54 from heptanoyl chloride (55) (14.9 g, 0.10 mol), thiophene (8.40 g, 0.10 mol), toluene (100 mL) and stannic chloride (10.4 g, 40 mmol). Distillation (b.p. 110°C/1 mm Hg) gave compound **37** (10.7 g, 55%) [Lit. value (54) 170–171°C/22 mm Hg, 84%].

7-(2′-Thienyl)-6-tridecene (**42**). A three-necked flask (1000 mL) was equipped with a dropping funnel, reflux condenser, thermometer and magnetic bar. The flask was charged with magnesium (14.9 g, 0.61 mol), a small amount of iodine and dry diethyl ether (60 mL). Hexyl bromide (100.9 g, 0.61 mol) in dry ether (130 mL) was added under stirring at such a rate that gentle reflux was maintained. When the addition was complete, the reaction mixture was refluxed for 20 min, dry ether (100 mL) was added and the reaction mixture was cooled in an ice bath. 2-Heptanoylthiophene (80.0 g, 0.41 mol) was added dropwise for 10 min, and the reaction mixture was refluxed for another 15 min, after which it was poured into a mixture of saturated ammonium chloride solution (800 mL) and ice (500 g) and stirred for 15 min. The product was extracted with ether (2 × 400 mL), and the combined ether phases were washed with water and dried over magnesium sulfate. After removal of the ether, the residue was treated with oxalic acid (4.0 g) and a small amount of hydroquinone (to avoid polymerization) and heated at 90°C for 30 min. The product was taken up in ether, and the ether solution was washed with water and sodium carbonate solution. After drying over magnesium sulfate and evaporation, the residue was distilled in an apparatus powdered with hydroquinone (b.p. 122°C/0.5 mm Hg) giving compound **42** (54.5 g, 42.5%). ^{1}H NMR ($CDCl_3$) δ 0.89 (*m*, 6H, CH_3), 1.20–1.38 (*m*, 14H, CH_2), 2.17 (*q*, 2H, CH_2), 2.45 (*t*, 2H, CH_2), 5.90 (*t*, 1H, C*H*), 6.95 (*m*, 2H), 7.08) *q*, 1H, J = 4.55 and 1.77 Hz). MS (EI) *m/e* (rel. int.) 264(8), 207(13), 179(22), 124(100), 97(57), 41(48).

7-(5′-Carboxy-2′-thienyl)-6-tridecene (**43**). Butyllithium (116 mL, 1.48 N) was added at room temperature under nitrogen and with magnetic stirring to 7-(2′-thienyl)-6-tridecene (**42**) in dry diethyl ether (350 mL). When the addition was complete, the reaction mixture was refluxed for 20 min, after which it was poured onto dry ice covered with dry ether. Water (560 mL) was added at 0°C, the phases were separated and the ether phase was extracted with sodium hydroxide solution (40 mL, 2N). The combined alkaline phases were acidified with sulfuric acid (2N) while cooling with ice. The product was dissolved in ether, the ether phase dried over magnesium sulfate and the ether evaporated to give compound **43** as an oil (44.0 g, 83%). MS after silylation (EI) *m/e* (rel. int.) 380(3), 295(7), 240(17), 193(8), 73(100), 43(10).

4-(2′-Thienyl)-3-decene (**38**) and 4-(2′-thienyl)-4-decene (**39**) were prepared analogously to **42** from magnesium (6.38 g, 0.26 mol) in dry diethyl ether (60 mL), propyl bromide (32.2 g, 0.27 mol) in dry ether (100 mL) and heptanoylthiophene (34.3 g, 0.175 mol). Distillation (b.p. 108–110°C/1.0 mm Hg) gave compounds **38** and **39** (24.7 g, 63%).

4-(5′-Carboxy-2′-thienyl)-3-decene (**40**) and 4-(5′-carboxy-2′-thienyl)-4-decene (**41**) were prepared analogously to **43** from **38** and **39** (24.7 g, 0.11 mol) in anhydrous diethyl ether (200 mL) and butyllithium (73.5 mL, 1.51 N). After evaporation, compounds **40** and **41** were obtained as an oil (23.2 g, 78%).

2-(2′-Thienyl)-1-octene (**44**) and 2-(2′-thienyl)-2-octene (**45**) were prepared analogously to **42** from magnesium (1.86 g, 76.4 mmol) in dry diethyl ether (20 mL), methyl iodide (10.8 g, 76.4 mmol) in dry ether (30 mL) and heptanoylthiophene (10.0 g, 50.9 mmol) in anhydrous ether (30 mL). Distillation (b.p. 92–96°C/1.5 mm Hg) gave compounds **44** and **45** (6.90 g, 70%).

2-(5′-Carboxy-2′-thienyl)-1-octene (**46**) and 2-(5′-carboxy-2′-thienyl)-2-octene (**47**) were prepared analogously to **43** from **44** and **45** (8.5 g, 43.5 mmol) in anhydrous diethyl ether (80 mL) and butyllithium (28.8 mL, 1.51 N). Upon acidification, one of the isomers, **46**, precipitated as yellow crystals (m.pl 65–68°C). After evaporation compounds **46** and **47** were obtained as an oil (5.85 g, 56%).

Decanoylthiophene (**48**) was prepared analogously to **37** from decanoyl chloride (56) (76.3 g, 0.40 mol), thiophene (33.6 g, 0.40 mol) in anhydrous toluene (400 mL) and stannic chloride (41.7 g, 0.16 mol). Distillation (b.p. 123°C/0.4 mm Hg) gave compound **48** (61.7 g, 64%) [Lit. value (57) b.p. 205°C/22 mm Hg].

6-(2′-Thienyl)-5-pentadecene (**49**) and 6-(2′-thienyl)-6-pentadecene (**50**) were prepared analogously to **42** from magnesium (15.7 g, 0.64 mol) in anhydrous diethyl ether (100 mL), pentyl bromide (97.0 g, 0.64 mol) in dry ether (120 mL) and decanoylthiophene 102.9 g, 0.43 mol) in anhydrous ether (300 mL). Distillation (b.p. 168°C/1.5 mm Hg) gave compound **49** and **50** (70.8 g, 56%).

6-(5′-Carboxy-2′-thienyl)-5-pentadecene (**51**) and 6-(5′-carboxy-2′-thienyl)-6-pentadecene (**52**) were prepared analogously to **43** from **49** and **50** (70.8 g, 0.25 mol) in anhydrous diethyl ether (400 mL) from butyllithium (193 mL, 1.42N). Upon evaporation, compounds **51** and **52** were obtained as an oil (78.1 g, 94%).

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The Relationship Between the Structure of Monoalkyl Branched Saturated Fatty Acids and Some Physical Properties

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The gel point and the refractive index of monoalkyl branched saturated fatty acids were determined and related to the structure of the acids. A three-factor central composite face-centered design was constructed where the three variables were the length of the main chain, the branching position and the length of the side chain. Second-order models were calculated in which the two properties were related to the structure. In order to confirm the validity of the models, two additional fatty acids were analyzed. The gel point was found to be proportional to the length of the main chain and the square of the branching position whereas the refractive index was proportional to the length of both the main and the side chain and also to the square of the main chain.
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It has long been known that the introduction of a branch into fatty acids and their derivatives alters their physical properties, *e.g.*, the melting point, the density, the refractive index and the viscosity (1-10). Many branched fatty acids, most of which are methyl branched, are found in nature (11-16).

In spite of the commercial interest in branched fatty acids there has, to our knowledge, only been one previous study in which the relationship between the structure of branched fatty acids and their physical properties has been systematically investigated. This is the study by Cason and Winans (9) in which all methyl branched octadecanoic acids, from 2-methyloctadecanoic acid to 17-methyloctadecanoic acid, were synthesized. The melting points were studied, and it was found that the melting points were all lower than for octadecanoic acid itself. The lowering effect was largest when the methyl group was attached to carbon number 10 or 11.

In the present study, two physical properties, the gel point ("solidification point") and the refractive index (at 80°C), of monoalkyl branched saturated fatty acids were related to the structure. A chemometrical approach was used. Polymorphic properties, if any, have not been considered.

MATERIALS AND METHODS

Materials. 2-Ethylhexanoic acid was purchased from E. Merck (Darmstadt, Germany). All other fatty acids were synthesized (17). The purity of the acids were found to be >98% by gas-liquid chromatography (GLC) and/or high-performance liquid chromatography (HPLC).

Measurements. The gel points were determined on a Bohlin VOR Rheometer (Lund, Sweden) used in the oscillation mode. The samples were placed between a cone and a plate with diameters of 25 mm and a gap size of 0.15 mm. The gap was adjusted at 0°C for the samples with high gel points (> −10°C) and at −25°C for the samples with low gel points (< −10°C). The torsion bar used had a torque constant of 92.3 gcm. The samples were subjected to an oscillation (frequency 0.500 s^{-1}) in the autostrain mode. This means that the strain was adjusted according to the measured torque in such a way that increased torque led to a decrease in strain. The strain amplitude varied between 0.002 and 0.2. The measurements were performed in a standard low temperature cell cooled by liquid nitrogen. The samples were cooled from −10 to −100°C (10 to −10°C for the acids with high gel points) with a temperature gradient of −0.5°C/15 s. The results were recorded every 6 s.

The gel points were determined by means of the tangent to the sharply increasing storage modulus (G′, the elastic component of a viscoelastic material). This indicates an increase in the elastic component of the sample (a solidification). The results are strongly dependent on the temperature gradient chosen, due to the fact that the acids show different polymorphic behavior. This method of determining the gel point is a modification of the method of Bohlin *et al.* (8).

The gel points of the acids with very high gel points (>10°C) were determined simply by agitating 2 g of the acid contained in a small bottle with a thermometer using the same frequency as in the rheometer. The samples were cooled by the surrounding air and, when necessary, with an ice-water mixture. The cooling was carried out in such a way as to achieve the same gradient as with the rheometer (−2°C/min).

The refractive indices were determined at 80°C with an Abbé refractometer, Carl Zeiss (Jena, Germany), model A.

Experimental design. The structures of the monoalkyl branched saturated fatty acids were chosen according to a three-factor central composite design (18). The three factors are the length of the main chain (X_1), the branching position (X_2) and the length of the side chain (X_3). As can be seen in Table 1, the different levels of variable X_2 are defined as functions of variable X_1. In a similiar way the levels of variable X_3 are defined as functions of variables X_1 and X_2. This is due to the fact that, *e.g.*, in the case of the branching position, level +1 represents a carbon atom near the end of the main chain. Depending on the length of the main chain, the number of the carbon atom to which

TABLE 1

Factor Levels of the Experimental Design

	Level		
Factor	−1	0	+1
Length of main chain (X_1)	6	12	18
Branching position (X_2)	2	$X_1/2$	$X_1 - 2$
Length of side chain (X_3)	1	$(X_1 - X_2 + 1)/2$	$X_1 - X_2$

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Abbreviations: ANOVA, analysis of variance; CCF, central composite face-centered design; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MODDE, modeling and design (computer software).

TABLE 2

Experimental Design Including Two Control Substances

Branched fatty acid	Length of main chain (X_1)	Branching position (X_2)	Length of side chain (X_3)
Acids included in the design			
2-Methylhexanoic	−1	−1	−1
2-Butylhexanoic	−1	−1	+1
4-Methylhexanoic	−1	+1	−1
4-Ethylhexanoic	−1	+1	+1
2-Methyloctadecanoic	+1	−1	−1
2-Hexadecyloctadecanoic	+1	−1	+1
16-Methyloctadecanoic	+1	+1	−1
16-Ethyloctadecanoic	+1	+1	+1
6-Propyldodecanoic	0	0	−0.2
9-Pentyloctadecanoic	+1	0	0
3-Ethylhexanoic	−1	0	0
10-Methyldodecanoic	0	+1	−1
2-Pentyldodecanoic	0	−1	−0.111
6-Hexyldodecanoic	0	0	+1
6-Methyldodecanoic	0	0	−1
Control acids			
6-Pentylpentadecanoic	+0.5	−0.273	0
2-Ethylhexanoic	−1	−1	−0.333

the branch is affixed will vary. In the same way the length of the branch has an upper limit. If this limit is passed, the branch has become the main chain. In the case of the branching position, level +1 has been chosen as a position two carbons away from the end of the main chain to be able to vary the length of the branch.

The complete experimental design, including two control substances, is shown in Table 2. As can be seen, the axial points have been placed one unit away from the center instead of the ideal distance of 1.682 units. This variant of the central composite design is called central composite face-centered design (CCF).

Statistical analyses. The evaluation of the results was made with the help of the software MODDE (MODeling and DEsign) version 1.2 (Umetri AB, Umeå, Sweden). All measurements were done in a randomized order. Measurements in the center point of the design were repeated twice. MODDE uses multiple linear regression to calculate a prediction equation for each response (Equation 1):

$$Y_p = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad [1]$$

The estimated coefficients are then rejected or retained depending on their probability. The test is performed at a significance level of $\alpha = 0.05$. The significant coefficients are then reestimated to give the final prediction equation. Outliers, identified by normal probability plots of the residuals (19), are excluded from the multiple linear regression.

RESULTS AND DISCUSSION

Gel point. The coefficients of the final prediction equation of the gel point are shown in Table 3 with corresponding probabilities and 95% confidence intervals. 9-Pentyloctadecanoic acid and 3-ethylhexanoic acid have been identified as outliers (by the technique of normal probability plots) and are consequently excluded from the regression calculations. The resulting equation is Equation 2.

TABLE 3

Coefficients from the Final Prediction Equations of the Gel Point and the Refractive Index

Term	Coefficient[a]	Probability[b]
Gel point		
Constant	−79 ± 13	0.00
Main chain (X_1)	68 ± 10	0.00
Branching position (X_2	0.5 ± 9	0.91
(Branching position)^2 (X_2^2)	69 ± 16	0.00
Refractive index		
Constant	1.4271 ± 0.0018	0.00
Main chain (X_1)	0.0146 ± 0.0015	0.00
Side chain (X_3)	0.0037 ± 0.0015	0.02
Main chain^2 (X_1^2)	−0.0075 ± 0.0024	0.00

[a]Values are given with 95% confidence intervals.
[b]The probability that a coefficient is equal to zero.

$$Y_{p,\ \text{gel point}} = -79 + 68X_1 + 0.5X_2 + 69X_2^2 \quad [2]$$

According to the prediction equation, the gel point is proportional to the length of the main chain (X_1) and to the square of the branching position (X_2^2). Any polymorphic properties, if present, have not been considered. Figure 1 shows a three-dimensional picture of the response surface, which has the shape of a long and rising valley.

The observed and the predicted gel points are compared in Table 4. An analysis of variance (ANOVA) scheme is shown in Table 5. The coefficient of multiple determination is $R^2 = 0.964$. The test for lack of fit indicates a high probability (40%) that the lack of fit is due to pure experimental error.

TABLE 4

Gel Points and Refractive Indices of Monoalkyl Branched Saturated Fatty Acids

Branched fatty acid	Gel point			Refractive index (80°C)		
	Observed gel point (°C)	Predicted gel point[a] (°C)	Residual (°C)	Observed refractive index	Predicted refractive index[a]	Residual
Acids included in the design						
2-Methylhexanoic	−89	−78 ± 17	−11	1.3970	1.4014 ± 0.0025	−0.0044
2-Butylhexanoic	−84	−78 ± 17	−6	1.4091	1.4088 ± 0.0025	0.0003
4-Methylhexanoic	−86	−77 ± 17	−9	1.4032	1.4014 ± 0.0025	0.0018
4-Ethylhexanoic	−57	−77 ± 17	20	1.4091	1.4088 ± 0.0025	0.0003
2-Methyloctadecanoic	53	57 ± 17	−4	1.4292	1.4305 ± 0.0025	−0.0013
2-Hexadecyloctadecanoic	73	57 ± 17	16	1.4391	1.4379 ± 0.0029	0.0012
16-Methyloctadecanoic	48	58 ± 17	−10	1.4319	1.4305 ± 0.0025	0.0014
16-Ethyloctadecanoic	51	58 ± 17	−7	—[b]	1.4379 ± 0.0029	—
6-Propyldodecanoic	−85	−79 ± 13	−6	1.4273	1.4264 ± 0.0018	0.0009
	−86	−79 ± 13	−7	1.4272	1.4264 ± 0.0018	0.0008
	−83	−79 ± 13	−4	1.4273	1.4264 ± 0.0018	0.0009
9-Pentyloctadecanoic	−77	−11 ± 17	−66	1.4329	1.4342 ± 0.0023	0.0013
3-Ethylhexanoic	−97	−146 ± 17	49	1.4069	1.4051 ± 0.0020	0.0018
10-Methyldodecanoic	−3	−9 ± 13	6	—[b]	1.4234 ± 0.0023	—
2-Pentyldodecanoic	−5	−10 ± 13	5	1.4257	1.4267 ± 0.0018	0.0010
6-Hexyldodecanoic	−86	−79 ± 13	−7	1.4280	1.4308 ± 0.0025	0.0028
6-Methyldodecanoic	−53	−79 ± 13	26	1.4245	1.4234 ± 0.0023	0.0011
Control acids						
6-Pentylpentadecanoic	−72	−40 ± 13	−32	1.4335	1.4325 ± 0.0016	0.0010
2-Ethylhexanoic	−83	−78 ± 17	−5	1.4011	1.4038 ± 0.0021	0.0027

[a]Values are given together with their 95% confidence intervals. [b]Insufficient material to perform the measurements.

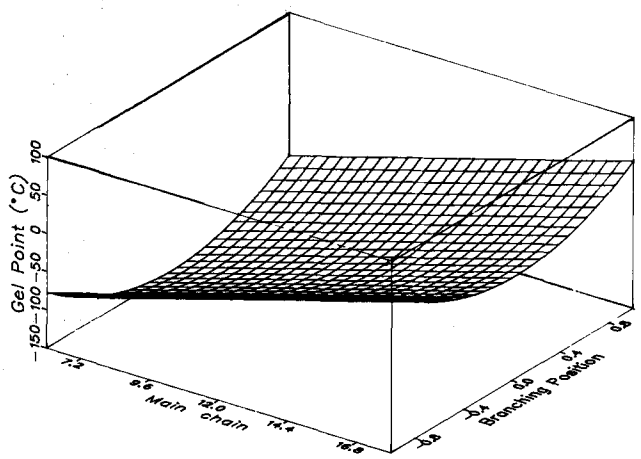

FIG. 1. Three-dimensional response surface showing the gel point as a function of the structure for monoalkyl branched saturated fatty acids. The length of the main chain (X_1) is given as the number of carbons (6 to 18) while the branching position (X_2) is given as coded values (−1 to +1).

TABLE 5

ANOVA Scheme and Residual Sum of Squares Breakdown for the Gel Point Prediction Equation[a]

Source of variation	ANOVA scheme: Sum of squares	Degrees of freedom	Mean square
Constant	16 138	1	16 138
Model terms	52 320	3	17 440
Residuals	1 956	11	178
Total	70 414	15	4 694
Residual sum of squares breakdown			
Pure error	1 463	8	183
Lack of fit	494	3	165

[a]P (lack of fit = pure error) = 0.400. ANOVA, analysis of variance.

The compounds for which the difference between the predicted and the observed gel point is largest have in common that they are branched near the middle ($X_2 \approx 0$) and that they, at the same time, have main chains which are not twelve carbons long. In other words the second order model does not give good predictions of the gel points of long or short fatty acids branched near the middle of the main chain. The lowering effect on the gel point achieved by putting a branch near the middle seems to be more pronounced the longer the main chain. This effect cannot be explained by the model. However, all other types of branched fatty acids, within the experimental domain, are satisfactorily explained.

The fact that the length of the branch does not influence the gel point came as a surprise. This is probably due to the fact that earlier investigations had been focused on methyl branched acids. Almost all naturally occurring branched fatty acids found up to now are methyl branched ones (2,3,11,14). On the other hand, the influence of the square of the branching position is a confirmation and extension of the results of the study on methyl branched octadecanoic acids by Cason and Winans (9).

Refractive index (80°C). The final prediction equation of the refractive index resulted in the coefficients shown

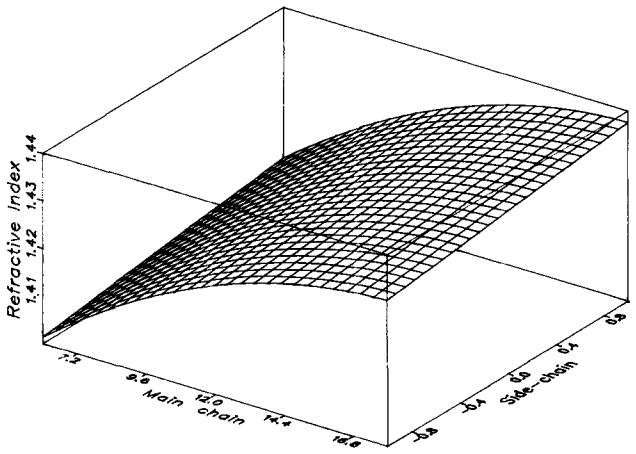

FIG. 2. Three-dimensional response surface showing the refractive index (at 80°C) as a function of the structure for monoalkyl branched saturated fatty acids. The length of the main chain (X_1) is given as the number of carbons (6 to 18) and the length of the side chain (X_3) is given as coded values (−1 to +1).

in Table 3 (with corresponding probabilities and 95% confidence intervals). The equation is Equation 3.

$$Y_{p,\ \text{refractive index (80°C)}} = 1.4271 + 0.0146X_1 + 0.0037X_3 - 0.0075X_1^2 \quad [3]$$

In this case the branching position does not influence the response. The refractive index is instead proportional to the length of both the main and the side chain and to the square of the length of the main chain.

Figure 2 shows a three-dimensional picture of the response surface. The conclusion is that the larger the molecule, the higher the refractive index. The observed and the predicted values are compared in Table 4. An ANOVA scheme is shown in Table 6. The coefficient of multiple determination is $R^2 = 0.980$, which is even better than in the gel point case. The test for lack of fit shows a high probability (44%) that the lack of fit is due to pure experimental error.

ACKNOWLEDGMENT

This work was financially supported by a grant from Karlshamns AB's Research Foundation.

TABLE 6

ANOVA Scheme and Residual Sum of Squares Breakdown for the Refractive Index Prediction Equation[a]

ANOVA scheme			
Source of variation	Sum of squares	Degrees of freedom	Mean square
Constant	30.2983	1	30.2983
Model terms	0.0022	3	0.0007
Residuals	0.0000	11	0.0000
Total	30.3005	15	2.0200
Residual sum of squares breakdown			
Pure error	2.24×10^{-5}	5	4.49×10^{-6}
Lack of fit	2.29×10^{-5}	6	3.81×10^{-6}

[a]*P* (lack of fit = pure error) = 0.439. ANOVA, analysis of variance.

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3-Carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene: A Site-Directed Inactivator of Yeast Oxidosqualene Cyclase

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The role and location of essential thiol groups in 2,3-oxidosqualene cyclase from *Saccharomyces cerevisiae* was examined (i) by comparing inactivation properties of two known thiol reagents, 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) and 2-nitro-5-thiocyanobenzoic acid (NTCB), with 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene (CNDT-squalene), a new thiol reagent designed as a site-directed inactivator of oxidosqualene cyclase and (ii) by testing the ability of the substrate to protect the enzyme against inactivation by the reagents. All reagents gave a time-dependent inactivation following pseudo-first order kinetics. DTNB and CNDT-squalene showed comparable inactivation ability (K_i = 0.67 and 1.21 mM), whereas NTCB was less effective (K_i = 15.6 mM). Strong differences between the two most active inhibitors, DTNB and CNDT-squalene, were observed when the enzyme was saturated with substrate prior to incubation with the thiol reagent. While substrate did not protect the enzyme against the inactivation caused by DTNB, a reduction in the inactivation ability of CNDT-squalene was observed under protection conditions. The data suggest that the squalene-like inactivator modifies a thiol group located at the active site of the enzyme.
***Lipids* 28, 903–906 (1993).**

Squalene and oxidosqualene cyclizing enzymes play a central role in the biosynthesis of steroid-like molecules, as they catalyze the conversion of acyclic precursors into tetra- or pentacyclic compounds (1,2). In recent years, several research groups have focused their efforts on the characterization and purification of these cyclizing enzymes from both prokaryotic and eukaryotic organisms (3–11). The evolutionary connection between these two classes of enzymes has been hypothesized on the basis of the ability of prokaryotic squalene cyclases to cyclize 3*R*- and 3*S*-2,3-oxidosqualene, in addition to their natural substrate, squalene (12). An important property shared by prokaryotic and eukaryotic cyclizing enzymes is their sensitivity to thiol reagents. *p*-Chloromercuribenzenesulfonic acid and *N*-ethyl maleimide strongly inhibit squalene-hopene cyclase from *Bacillus acidocaldarius* (13) and oxidosqualene-lanosterol cyclase from yeast (9) and hog liver (6). In the latter work cited, the authors demonstrated that a sulfhydryl group is essential for the activity of mammalian liver cyclase, using inactivation experiments with *N*-ethyl maleimide as a modifying agent. However, the presence of a reactive thiol group at the active site of the enzyme could not be proven, as no protection against inactivation was observed when experiments were repeated in the presence of a competitive inhibitor as a protecting agent (6).

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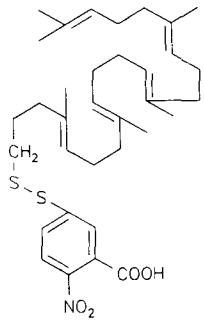
Abbreviations: CNDT-squalene, 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; TNB, thionitrobenzoate.

The search for more specific inactivators of yeast oxidosqualene cyclase, which would be able to enter and modify the active site of the enzyme, led us to design new thiol modifying agents with squalene-type substituents. In the present paper we desdribe a chemical modification of yeast oxidosqualene cyclase by 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene (CNDT-squalene) (Scheme 1), a new thiol reagent designed as a site-directed inactivator. Properties of the new reagent were compared with those of the two known thiol reagents, 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB), the Ellman's reagent and 2-nitro-5-thiocyanobenzoic acid (NTCB), which inactivates proteins by cyanylating thiol groups (14). Protection experiments, performed with the new reagent, provided the first evidence of the existence of an essential thiol group within the active site of the enzyme.



SCHEME 1

MATERIALS AND METHODS

Chemicals. Nonidet P-40, Tween 80, NTCB and DTNB were obtained from Sigma Chimica (Milan, Italy). 2,3-Oxidosqualene and [3-^{3}H]2,3-oxidosqualene (44 × 10^6 dpm/μmol) were synthesized as reported (15). CNDT-squalene was synthesized (16) by an exchange reaction between 5,5′-dithio*bis*(2-nitrobenzoic acid) and 1,1′,2-*tris*norsqualene thiol (17).

Solubilization of 2,3-oxidosqualene cyclase from yeast microsomes. Saccharomyces cerevisiae (ATCC 12341) was cultured as previously described (18). The yeast homogenate, obtained by sonication of washed cells in 1 vol of 0.1 M Na/K phosphate buffer (pH 7.1) (10,18), was cleared up by low-speed centrifugation (10,000 × *g* for 15 min) and then centrifuged at 150,000 × *g* for 90 min. The pellet was resuspended in 0.1 M phosphate buffer (pH 7.1) at 4°C, and a solution of Nonidet P-40 in the same buffer was added dropwise to arrive at a final concentration of protein and detergent of 2 and 1 mg/mL, respectively. The solution was gently stirred for 60 min at 4°C and then centrifuged at 150,000 × *g* for 60 min. Solubilized proteins (0.25 to 0.35 mg/mL) had a specific activity ranging from 40 to 60 nmol lanosterol/h/mg protein. At 35°C, spontaneous decay of enzyme activity from different preparations

ranged from 80 to 60% of the original activity within 30 min.

2,3-Oxidosqualene cyclase assay. The assay procedure used was essentially as previously reported (10). Briefly, the reaction mixture containing [^{3}H]2,3-oxidosqualene (70,000 dpm), Tween 80 0.2 mg/mL and solubilized microsomes (0.3 mg prot/mL) in 0.1 M Na/K phosphate buffer (pH 7.1), was incubated for 30 min at 35°C. The nonsaponifiable extract was separated by thin-layer chromatography, and bands co-migrating with 2,3-oxidosqualene and lanosterol were monitored by liquid scintillation counting.

Time-dependent inactivation; general procedure. The enzyme solubilized from microsomes with detergent in 0.1 M phosphate buffer, pH 7.1, was incubated at 35°C with different concentrations of reagent. After the preincubation period, the inactivator was removed by 10-fold dilution of the enzyme preparation or by direct neutralization of the thiol reagent with dithiothreitol or mercaptoethanol (both ineffective on oxidosqualene cyclase at up to 20 mM). Enzyme activity was then assayed as described above. Either way of removing the inactivator after the preincubation gave similar results. Differences in the spontaneous decay of the enzyme activity can be observed dependent on the conditions under which the inhibitors were solubilized.

Protein determination. Proteins were determined by the method of Lowry, as modified by Peterson, using bovine serum albumin as a standard (19).

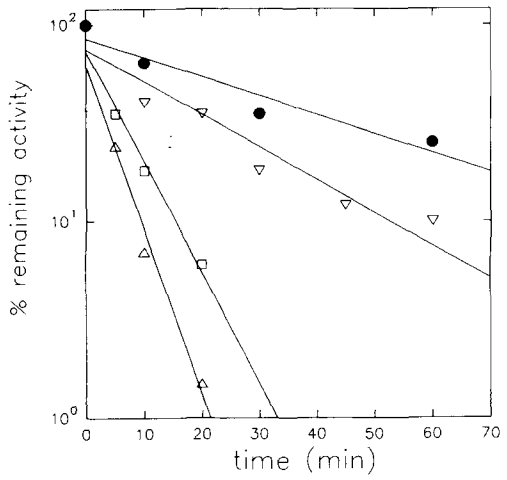


FIG. 1. Time-dependent inactivation of yeast oxidosqualene cyclase by 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene. The enzyme solubilized from microsomes with Nonidet P-40 was incubated (0.3 mg protein/mL, 0.1 M K/Na phosphate buffer, pH 7.1, containing 1 mg/mL Nonidet P-40) at 35°C with 0.0 (●), 0.1 (▽), 0.5 (□) and 1 mM (△) reagent. At time intervals, 50 μL-aliquots were withdrawn and diluted with 450 μL 0.1 M K/Na phosphate buffer, pH 7.1, containing 0.5 mg/mL Nonidet P-40, 0.2 mg/mL Tween 80, [^{3}H]2,3-oxidosqualene (50,000 dpm) and 2,3-oxidosqualene (25 μM). The mixture was then incubated for 30 min at 35°C to determine the residual activity, which is expressed as percentage of control (no reagent).

RESULTS AND DISCUSSION

Inactivation of yeast oxidosqualene cyclase by CNDT-squalene, NTCB and DTNB. The ability of CNDT-squalene, NTCB and DTNB to inactivate yeast oxidosqualene cyclase was evaluated by measuring the enzyme activity solubilized from microsomes after exposure to each of the thiol reagent at different concentrations. In all cases, a time-dependent inactivation following pseudo-first order kinetics was observed (Figs. 1 and 2). The similarity of the kinetic patterns was also apparent by comparison of the reciprocal plots of the pseudo-first order rate constants *vs.* the reagent concentratons (Figs. 3 and 4). All reagents showed saturation kinetics indicating that an enzyme/reagent reversible complex is formed prior to the inactivation event (20). Apparent binding constants, K_i (mM), and inactivation rate constants at saturation, $kapp_{max}$ (min^{-1}), for each reagent were calculated from the slope and intercept of the linear plot, respectively (Table 1). CNDT-squalene and DTNB inactivated oxidosqualene cyclase faster than did NTCB (Table 1).

Effect of the substrate on the rate of inactivation by CNDT-squalene, NTCB and DTNB. In order to localize the thiol groups involved in the inactivation of oxidosqualene cyclase, substrate protection experiments were performed in the presence of the three inactivators of the enzyme. In the only reported attempt to protect an oxidosqualene cyclizing enzyme against thiol reagents, no protection was observed (6). In the cited work, the authors reacted the pig liver enzyme with *N*-ethyl maleimide in the presence of a competitive inhibitor of oxidosqualene cyclase. The lack of protection suggested that the sulfhydryl group modified by the inactivator was not directy involved in the catalytic process, but played a role in the maintenance

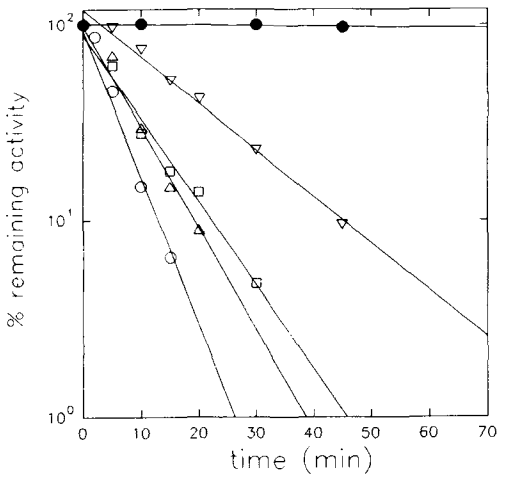


FIG. 2. Time-dependent inactivation of yeast oxidosqualene cyclase by 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB). Treatment of the enzyme and incubation conditions were as described in the legend for Figure 1. DTNB concentrations during inactivation time: 0.0 (●), 0.1 (▽), 0.3 (□), 0.5 (△) and 0.6 mM (○).

of an active enzyme conformation. In our experiments, done with substrate as protecting agent, considerable differences were observed among the three inactivators. The substrate did not protect at all against inactivation caused by DTNB (Fig. 5), whereas a weak protection seemed to be detectable in experiments with the less powerful inhibitor NTCB. So far, however, data obtained with this weak inhibitor are not sufficient to conclude that the small and uncharged cyanogroup of the reagent modifies a thiol group within the active site of the enzyme. Better evidence

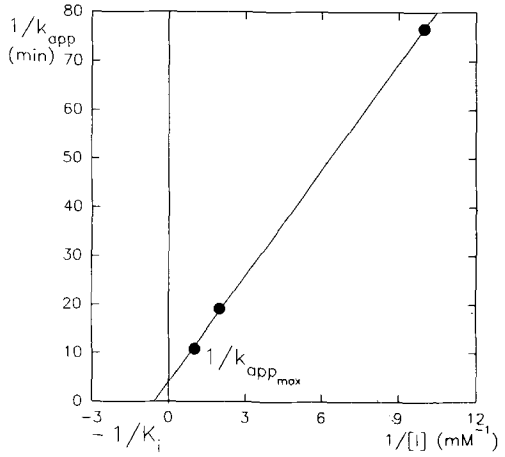


FIG. 3. Double reciprocal plot of the apparent pseudo-first-order rate constants (kapp) of inactivation *vs.* 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene concentrations; kapp were obtained from graph in Figure 1.

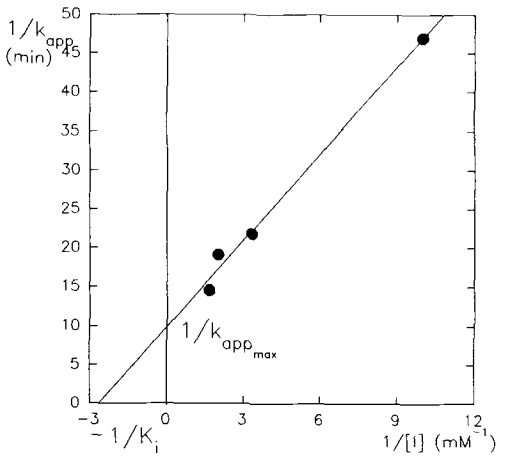


FIG. 4. Double reciprocal plot of the apparent pseudo-first-order rate constants (kapp) of inactivation *vs.* 5,5′-dithio*bis*(2-nitrobenzoic acid) concentrations; kapp were obtained from graph in Figure 2.

for the existence of a thiol group directly involved in the catalytic process came from protection experiments in the presence of CNDT-squalene: substrate, at a saturating concentration, clearly protected the enzyme against inactivation (Fig. 6). As CNDT-squalene molecules differ from the Ellman's reagent (DTNB) by a squalenoid residue in place of a nitrobenzoic residue, we suggest that the squalene moiety of the CNDT-squalene acted as a "delivery moiety," able to lead the modifying agent into the active site of the enzyme. The results were confirmed by following photometrically the release of TNB during the inactivation of the enzyme by DTNB and CNDT-squalene (21). While the release of TNB from DTNB was not affected by saturating substrate concentrations, there was a significant reduction of release of TNB from CNDT-squalene under the same conditions (Fig. 7). Other CNDT-derivatives, bearing different alkyl (aliphatic or isoprenic) moieties are at the moment under investigation for their inactivation properties. Preliminary results indicate that replacement of a squalene residue by a dodecane residue

TABLE 1

Inactivation of Yeast Oxidosqualene Cyclase by CNDT-squalene, DTNB and NTCB

Inactivator	Apparent binding constant[a] K_i (mM)	Inactivation rate constant[a] at [I] = ∞ $kapp_{max}$ (min^{-1})
CNDT-squalene	1.21	0.220
DTNB	0.67	0.093
NTCB	15.60	0.067

[a]K_i and $kapp_{max}$ were calculated from graphs similar to those shown in Figures 3 and 4. Values were corrected for the spontaneous decay of the enzyme. Abbreviations: CNDT-squalene, 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene; DTNB, 5,5′-dithio*bis*(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid.

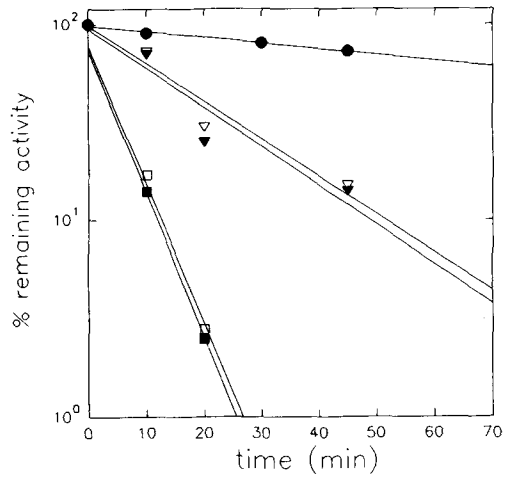


FIG. 5. Lack of protection by oxidosqualene (substrate) against 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) inactivation of yeast oxidosqualene cyclase. The enzyme solubilized from microsomes with Nonidet P-40 was pre-equilibrated (0.3 mg protein/mL, 0.1 M K/Na phosphate buffer, pH 7.1, containing 1 mg/mL Nonidet P-40 and 0.2 mg/mL Tween 80) at 35°C with substrate at a saturating concentration (0.5 mM) for 15 min. After addition of 0.0 (●), 0.1 (▼) and 0.5 mM (■) DTNB in ethanol solution, the experiment proceeded under conditions similar to those described for a normal inactivation experiment (see legend to Fig. 1). The residual activity was determined at 50 μM substrate. In control experiments performed without protection [inactivator concentrations: 0.1 (▽) and 0.5 mM (□)], substrate (50 μM) was added just before determination of the residual activity.

makes the inhibitor less specific and less active (G. Balliano, personal communication).

Results obtained with CNDT-squalene are consistent with the hypothesis of the existence of a cysteinyl residue essential for catalysis at the active site of yeast oxidosqualene cyclase. This residue could act as a protonating group in the acid-catalyzed opening of the oxirane ring of squalene-oxide. Further insights in the different classes of thiol groups of the yeast oxidosqualene cyclase should be achieved when it will be possible to repeat inactivation experiments with the enzyme purified to homogeneity.

ACKNOWLEDGMENTS

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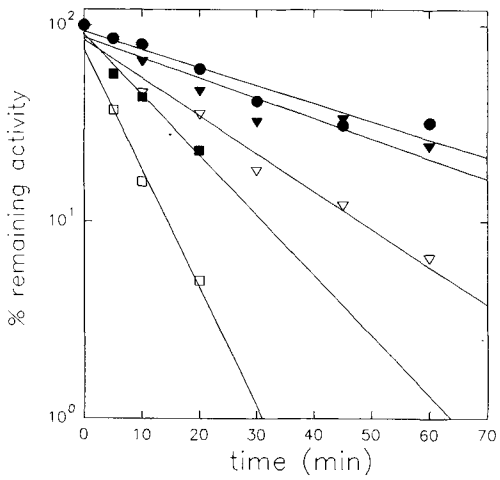

FIG. 6. Protection by oxidosqualene (substrate) against 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene (CNDT-squalene) inactivation of yeast oxidosqualene cyclase. Experiments were carried out as described in the legend to Figure 5. CNDT-squalene concentrations were 0.0 (●), 0.1 (▼) and 0.5 mM (■) in experiments with substrate (0.5 mM) as a protecting agent, and 0.1 (▽) and 0.5 mM (□) in experiments with no substrate as protecting agent.

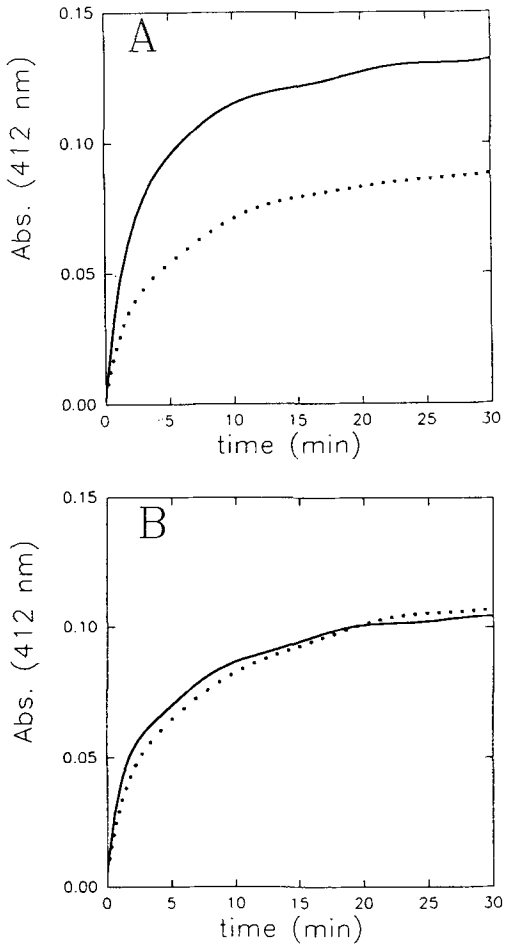

FIG. 7. Photometrical evaluation of protection against inactivation. The effect of substrate (0.5 mM) on the inactivation of yeast oxidosqualene cyclase by 3-carboxy-4-nitrophenyl-dithio-1,1′,2-*tris*norsqualene (CNDT-squalene) or 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) was evaluated by following photometrically, at 412 nm, the release of thionitrobenzoate during the reaction of the enzyme preparation (0.3 mg protein/mL in 0.1 M K/Na phosphate buffer, pH 7.1, containing 1 mg/mL Nonidet P-40) with 0.1 mM CNDT-squalene (A) or 0.1 mM DTNB (B). Dotted lines describe reactions in the presence of substrate as a protecting agent. Solid lines describe reactions in the absence of substrate. Abs., absorbance.

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General Resistance to Sterol Biosynthesis Inhibitors in *Saccharomyces cerevisiae*

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Screening for resistance to fenpropimorph was undertaken in order to isolate yeast mutants affected in the regulation of the ergosterol pathway. Among the mutants isolated, one bearing the recessive *fen1-1* mutation was characterized by a 1.5-fold increase in the ergosterol level and a general resistance to sterol biosynthesis inhibitors. The *fen1-1* mutation was linked to *MAT* locus on chromosome III. The measurement of enzyme activities involved in the ergosterol pathway revealed that isopentenyl diphosphate (IPP) isomerase activity was specifically increased 1.5-fold as compared to the wild type strain. However, overexpression of IPP isomerase in the wild type strain was not by itself sufficient to lead to sterol increase or resistance to sterol biosynthesis inhibitors, showing that IPP isomerase is not a limiting step in the pathway. The *fen1-1* mutation permits viability in aerobiosis of yeast disrupted for sterol-14 reductase in absence of exogenous ergosterol supplementation, whereas the corresponding strain bearing the wild type *FEN1* allele grows only in anaerobiosis. This result shows that ignosterol is able to efficiently replace ergosterol as bulk membrane component and that the *fen1-1* mutation eliminates the specific ergosterol requirement in yeast.
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The sterol biosynthetic pathway is the target of powerful fungicides. Among them, the most commonly used against pathogenic fungi are azoles and morpholine derivatives. Azoles, such as ketoconazole, flusilazol or LAB 170250 F, inhibit the C-14 lanosterol demethylase step (1-3) through the binding of the fungicide's heterocyclic nitrogen atom to the protohaem iron of cytochrome P450 lanosterol demethylase (4). Morpholines block two later steps downstream in the ergosterol pathway, Δ^{14} sterol reductase and Δ^{8}-Δ^{7} sterol isomerase (5). These compounds, which are likely to be protonated at physiological pH, share some structural and electronic similarities with the sterol carbocationic high energy intermediates involved in the Δ^{14} sterol reductase and Δ^{8}-Δ^{7} sterol isomerase reactions (6).

Ergosterol plays a specific role in yeast which has been described as regulatory (7), synergistic (8) or as sparking effect (9). In addition to ergosterol, a few ergosterol precursors have also been described to fullfil the sparking function (10). We have shown recently that fenpropimorph inhibits cell growth by leading to a specific drop in ergosterol level below the level required for the sparking function (11).

Regulation mechanisms of ergosterol biosynthesis in yeast are not well understood. Although hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase is probably implicated in the regulation (12), there is no definitive evidence that this enzyme is regulated by the ergosterol level. Specific activities of acetoacetyl-CoA thiolase, HMG-CoA synthase, squalene synthase and squalene epoxidase have been shown to be dependent on the sterol needs of the cells (13,14), but it is unlikely that ergosterol itself is the regulatory molecule (13). An approach to find out the limiting step or the key regulatory step in a pathway is the isolation and the characterization of mutant strains that overproduce the end product. The isolation of mutants resistant to an inhibitor specific for the pathway studied has been shown to be a valuable approach (15). In this report, we describe a mutant strain of *Saccharomyces cerevisiae* that was isolated and shown to be resistant to fenpropimorph. The main features of this strain are an overproduction of ergosterol and a strong resistance to a large number of sterol biosynthesis inhibitors. This paper describes a study on the mechanism of resistance displayed by this strain and its relation to ergosterol biosynthesis.

EXPERIMENTAL PROCEDURES

Strains and growth conditions. The strains used were derived from *S. cerevisiae* FL100 (ATCC 28383) haploid, a mating type, or from an isogenic strain FL200 (ATCC 32119) haploid, α mating type. *Saccharomyces cerevisiae* strain DD21 is a derivative of the Gal^{+} strain (16) W303-1B (*a* mating type, *leu2-3, 112, his3-11, 15, trp1-1, ura3-52, ade2-1, can1-100*).

Culture conditions and media were those previously described (11).

Stock solutions of fungicides were prepared as follows: fenpropimorph and fenpropidin (BASF, Agrochemical Station, Limburgerhof, Germany) and SF 86327 (Sandoz Forschungsinstitut, Wien, Austria), in ethanol (the final solvent concentration in the culture medium did not exceed 1%, vol/vol), ketoconazole (Janssen Pharmaceutica Bersee, Belgium), fluzilasol (Du Pont de Nemours and Co., Wilmington, DE) and LAB 170250 (BASF, Agrochemical Station) in sterile water. Amphotericin B and nystatin (Laboratoires Squibb, Paris, France) in sterile water containing 20% (vol/vol) dimethylformamide (DMF).

For the determination of MIC values (minimum inhibitory concentration inhibiting growth completely after 48 h incubation at 28°C) of drugs, approximatively 10^{6} cells were spotted on solid media which contained varying concentrations of the drug being tested.

Strain DD21 construction. A polymerase chain reaction (PCR) was carried out on yeast genomic DNA with two

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Abbreviations: ATP, adenosine triphosphate; cM, centimorgan (unit of map distance for gene-gene linkage); DMF, dimethyl formamide; HMG-CoA, hydroxymethylglutaryl-coenzyme A; IPP, isopentenyl diphosphate; FAD, flavine adenine dinucleotide; FPP, farnesyl diphosphate; GC, gas chromatography; GPP, geranyl diphosphate; MIC, minimal inhibitory concentration; MVA, mevalonic acid; PCR, polymerase chain reaction; pDD1 plasmid carrying yeast IPP isomerase gene; SBI, sterol biosynthesis inhibitor(s).

oligonucleotide primers derived from the yeast isopentenyl diphosphate (IPP) isomerase gene sequence (17). The PCR product was purified, digested with *Eco*RI and *Bam*HI and ligated into the *Eco*RI/*Bam*HI site of pYeDP1/8-2 (yeast replicative vector carrying p*GAL10-CYC1* promoter/*PGK* terminator 16). The resulting IPP isomerase expression vector (pDD1), recovered after propagation in *E. coli* strain 71/18, was used to transform yeast strain W303-1B giving DD21.

Total sterol extraction and analysis. Freeze-dried cells were saponified by the method of Molzahn and Woods (18). Sterols were extracted and analyzed by gas chromatography (GC) as described (11). Sterols with 5,7-diene system and 8.14-diene system were identified and quantified by their specific absorption spectra (19).

Separation of free and esterified sterols. The free sterol and steryl ester fractions were extracted from freeze-dried cells by a procedure adapted from Taylor and Parks (20). Free sterols and steryl esters were separated on a column of alumina (neutral, grade 1; Sigma Chimie, L'Isle D'Abeau, France) washed with *n*-hexane. Steryl esters were eluted with dichloromethane, then free sterols with ethanol.

Determination of radioactive [^{14}C-methyl] *methionine incorporation into sterols.* Cells (logarithmic growth phase) were cultured in minimum medium; aliquots (0.2 mL, approximately 3×10^7 cells) of the cultures were incubated with [^{14}C-*methyl*]methionine (0.15 mCi/mmol, 0.67 mM final concentration; Amersham, Les Ulis, France). Incubation was stopped by addition of 1 mL of 40% KOH (wt/vol). This mixture was heated at 60°C for 1 h. Nonsaponifiable lipids were extracted with *n*-heptane. After concentration under nitrogen, an aliquot was assayed for radioactivity by liquid scintillation counting. Incorporation of radioactivity was linear during 1 h.

Incorporation of [^{14}C]*mevalonic acid (MVA) or* [^{14}C]*IPP* in vitro. Cells were harvested during the exponential growth phase and washed thrice in ice-cold 0.05M Tris-HCl, pH 7.4, and then disrupted in the same buffer containing 1 mM dithiothreitol. The standard mixture contained in a final volume of 0.2 mL 10 mM $MgCl_2$, 20 mM adenosine triphosphate (ATP), 0.5 mM NADPH, 1 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, and [2-^{14}C](R)MVA (37.5 μM, 66.6 μCi/mmol; Amersham) or [1-^{14}C]IPP (40 μM; 1 mCi/mmol, Amersham); ATP was omitted in the incubation with IPP. Incubation temperature was 37°C. One volume of methanol was then added. Proteins were precipitated by heating at 65°C for 5 min and separated from the supernatant by centrifugation. Radioactivity in the supernatant [MVA to farnesyl diphosphate (FPP)] and in the pellet (nonsaponifiable fraction) was measured on aliquots. Reaction products in the pellet were identified by thin-layer chromatography using silica gel G plates developed with dichloromethane. The major compound was squalene, as expected, since flavine adenine dinucleotide (FAD) required for squalene epoxidase reaction was omitted. Incorporations were linear for 1 h in the range of protein concentration used (100–400 μg).

Enzyme preparation. Cells were harvested during the exponential phase of growth and washed with ice-cold 0.1M Tris-HCl buffer, pH 7.4. Cells were disrupted in the same buffer containing 5 mM dithiothreitol for IPP isomerase and squalene synthease, or 5 mM iodoacetamide for FPP synthase. A 12,000 × *g* supernatant was used as a crude extract. In studies of squalene synthase, we used a 105,000 × *g* microsomal fraction.

Protein concentration was determined by the biuret procedure using crystalline bovine serum albumin as a standard.

IPP isomerase assay. The standard mixture (21) contained in a final volume of 0.2 mL 100 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, and crude extract. The assay was initiated with 60 μM [1-^{14}C]IPP (1 mCi/mmol) and incubated for 5 min at 37°C. The sample was chilled on ice and 1 mL pentane was added, followed by 0.6 mL HCl, 0.33N. The mixture was shaken during 15 min at 37°C. After vigorous mixing, the two phases were allowed to separate. An aliquot (0.1 mL) of the pentane layer was assayed for radioactivity by liquid scintillation counting. Specific activity was expressed in nmoles IPP incorporated into acid-labile products per min per mg protein. Product formation at 37°C was proportional to protein amounts within 20–200 μg and to time within 2–8 min.

FPP synthase assay. The enzyme activity was assayed as previously described (22) with geranyl diphosphate (GPP) as the second substrate. Specific activity was expressed in nmol IPP incorporated into acid-labile products per min per mg protein.

Squalene synthease assay. The standard assay was the same as described by M'Baya *et al.* (14). Squalene synthase activity was determined as the radioactivity in the squalene fraction and expressed in nmol of FPP converted per min per mg protein.

RESULTS

Isolation of the fen1-1 *mutation.* We isolated one spontaneous and seven ultraviolet (UV)-induced mutants resistant to fenpropimorph. Genetic analysis showed that all mutations were recessive and belonged to seven complementation groups. Among them we studied the mutant strain FD49, bearing the *fen1-1* mutation, which showed a strong fenpropimorph resistance and an increased ergosterol level. On the contrary, all the other mutants showed a sterol composition similar to that of the wild type strain.

Phenotypic analysis. The MIC of different drugs was determined for the wild type strain and the mutant strain FD49. Table 1 shows that the mutant strain FD49 resists up to 66 μM fenpropimorph, while the wild type strain is inhibited by 0.3 μM fenpropimorph. A strong resistance to fenpropidin (piperidine), to SF 86327 (allylamine), to Lab 170250F, to ketoconazole and to flusilazol (azoles) was also observed. Nevertheless the FD49 mutant strain exhibited the same sensitivity as the wild type strain to various growth inhibitors such as polyene antibiotics (nystatin and amphotericin B), allylic alcohol, copper and cycloheximide. This result strongly suggests that the cross resistance of FD49 is specific to ergosterol biosynthesis inhibitors and therefore is not the result of a decrease in membrane permeability.

Genetic analysis. The heterozygous diploid obtained by crossing FD49 with the wild type strain was sensitive to fenpropimorph showing that the *fen1-1* mutation is recessive. Tetrad analysis showed a 2:2 segregation of resistance to sensitivity. The two resistant spores always showed a 40% increase in the level of sterols with a

TABLE 1

Effects of Growth Inhibitors on the Wild Type Strain and the Mutant Strain FD49[a]

Inhibitor	Strains	
	FL100	FD49
Fenpropimorph	0.3	>66
Fenpropidin	0.3	>66
SF86327	340	>680
Lab170250F	1.6	>16
Ketoconazole	6	>28
Flusilazol	3	>50
Nystatin	1	1
Amphotericin B	0.3	0.3
Copper	4800	4800
Cycloheximide	71	71
Allylic alcohol[b]	4370	4370

[a]The concentration of inhibitor which prevented growth on complete medium after 48 h of incubation was designated as the minimum inhibitory concentration and expressed in μM.
[b]Glycerol is the carbon source instead of glucose.

5,7-diene system (Table 2). These results show that a single mutation confers at the same time resistance to fenpropimorph and an increase in the sterol level.

Furthermore, it should be noted that recombinants bearing the *fen1-1* mutation exhibited, as did FD49, a 66% increase in generation time as compared to the wild type strain (Table 2). Tetrad analysis revealed that the *fen1-1* mutation was linked to the *MAT* locus (Table 3). To establish a genetic map, crosses were made with a strain bearing a *thr4* mutation localized at 22 cM from *MAT* locus (23). Results (Table 3) showed that the *fen1-1* mutation is located on chromosome III, at 6.6 cM from *MAT*, and 30 cM from *thr4*.

Sterol analysis. Sterols from both the FD49 mutant and the wild type strain were analyzed by GC to assess the

TABLE 2

Tetrad Analysis of Diploid FD49/FL100

Tetrad	MAT[a]	Phenotype[b]	Ergosterol level[c]	Mgt[d]
1 sporeA	α	+	1.00	2.5
sporeB	a	0	0.70	1.5
sporeC	a	0	0.72	1.5
sporeD	α	+	0.97	2.5
2 sporeA	α	+	1.00	2.5
sporeB	α	+	0.98	2.5
sporeC	a	0	0.72	1.5
sporeD	a	0	0.74	1.5
3 sporeA	α	+	1.00	2.5
sporeB	a	0	0.71	1.5
sporeC	a	0	0.72	1.5
sporeD	α	+	0.96	2.5
4 sporeA	a	0	0.68	1.5
sporeB	α	+	1.02	2.5
sporeC	α	+	1.07	2.5
sporeD	a	0	0.71	1.5

[a]Mating type.
[b]Phenotype: growth (+), or absence of growth (0), in medium containing 30 μM fenpropimorph.
[c]Ergosterol level is expressed as % of cell dry weight (mean of two independent determinations).
[d]Mgt: mean generation time in hours is approximate (±15 min).

TABLE 3

Genetic Mapping of *fen1-1* Mutation[a]

Interval	Ascus type			Map distance (cM)
	PD	NPD	T	
fen1-MAT	46	0	7	6.6
fen1-thr4	12	0	18	30.0
MAT-thr4	14	0	16	26.7

[a]Tetrads were isolated by ascus dissection, followed by mating type and phenotype determinations; cM, centimorgan (unit of map distance for gene-gene linkage); PD, parental ditype; NPD, nonparental ditype; T, tetratype.

effect of the *fen1* allele on sterol synthesis in the absence and presence of fenpropimorph. First we studied the sterol composition of both strains in the absence of the sterol biosynthesis inhibitor (SBI). The results showed (Tables 4 and 5) that the high level of sterols with a 5,7-diene system in FD49 is due to a specific increase in the ergosterol level. Treatment of wild type cells with increasing concentrations of fenpropimorph induced a modification of the sterol profile (Table 4) consisting in a progressive

TABLE 4

Sterol Composition of Fenpropimorph-Treated Wild Type Strain[a]

Sterol[b]	Fenpropimorph (μM)		
	0	0.066	0.660
Zymosterol	9.3	14	4.2
Ergosterol	57.1	16.6	—[c]
Fecosterol	4.3	10.9	—
Ignosterol	—	—	48.8
Ergost-8-enol	—	57.6	31.0
Ergosta-5,7-dienol	26.6	—	—
Lanosterol	2.7	0.9	15.5

[a]Cells were cultured for 15 h in complete medium containing different concentrations of fenpropimorph.
[b]The sterols were quantified by gas chromatography using the area method and expressed as % of total sterols. Cholesterol was used as internal standard.
[c]—, Not detected.

TABLE 5

Sterol Composition of Fenpropimorph-Treated FD49 Mutant Strain[a]

Sterol[b]	Fenpropimorph (μM)		
	0	0.066	0.660
Zymosterol	4.8	14.6	2.9
Ergosterol	73.3	1.4	—[c]
Fecosterol	4.4	—	—
Ignosterol	—	56.4	78.1
Ergost-8-enol	—	25.2	16.0
Ergosta-5,7-dienol	14.2	—	—
Lanosterol	3.3	2.4	3

[a]Cells were cultured for 15 h in complete medium containing different concentrations of fenpropimorph.
[b]The sterols were quantified by gas chromatography using the area method and expressed as % of total sterols. Cholesterol was used as internal standard.
[c]—, Not detected.

disappearance of ergosterol and in an accumulation of unusual sterols, such as ergost-8-enol and ergosta-8,14-dienol (ignosterol). At a 0.066 μM fenpropimorph concentration, which does not inhibit cell growth, the ergosterol content in cells was lowered by about 75%, and the major sterols were ergost-8-enol and fecosterol. At a concentration of 0.660 μM, cell growth was strongly inhibited, ergosterol was no longer detected and ignosterol accumulated in addition to ergost-8-enol (Table 4). The same progressive inhibition of ergosterol formation was observed in FD49 (Table 5), but, surprisingly, ignosterol levels increased simultaneously with ergost-8-enol levels at a fenpropimorph concentration as low as 0.066 μM. In contrast, the wild type strain accumulated ignosterol only at a much higher drug concentration (*i.e.*, 0.660 μM). Treatment with fenpropimorph induces an accumulation of the same sterols in FD49 and the wild type strain. Therefore, resistance mechanisms, such as failure in drug uptake, drug expulsion, detoxification, or a decrease in affinity of the target enzymes for the inhibitor, can be ruled out. It has been suggested (24,25) that antifungal activity of morpholines and piperidines is directly related to the accumulation of ignosterol; its integration into membranes is thought to disorganize membrane structure due to the presence of the 8,14-diene system. Since only free sterols are incorporated into membranes while esterified sterols are largely stored as lipid droplets (20,26), an increased degree of esterification of unnatural sterols could result in resistance to fenpropimorph. We measured free and esterified sterol levels in FD49 and FL100 in order to check this hypothesis. The results (Table 6) showed that both strains displayed the same strong degree of esterification of the atypical sterols, ergosta-8-enol and ergosta-8,14-dienol, while ergosterol essentially existed in the free state. Therefore, esterification does not explain the resistance properties of FD49. Moreover, since the levels of free ignosterol and ergost-8-enol are similar in both strains, the toxicity of fenpropimorph could not be due to the presence of these sterols in membranes. This also agrees with the hypothesis (11) that specific ergosterol starvation is at the basis of cell growth inhibition by morpholines. Consequently, the resistance mechanism might be directly related to the increase of ergosterol observed in FD49.

Ergosterol metabolism in FD49 and wild type strains. *In vivo* measurement of ergosterol biosynthesis using [^{14}C-*methyl*]methionine showed, as expected, that the rate of sterol biosynthesis is increased about 1.6-fold in the mutant strain FD49 in comparison with the wild type strain (Table 7). In order to identify enzymatic steps responsible for this increase, we measured the *in vitro* transformation of both MVA and IPP into nonsaponifiable lipids; transformations of the two intermediates were again increased 1.5-fold in FD49 in terms of specific activity as shown in Table 6. This result clearly shows that a specific enzyme activity, downstream from IPP formation, is increased in FD49, as compared to the wild type strain. We measured the specific activities of IPP isomerase, FPP synthase and squalene synthase. Table 8 shows that IPP isomerase specific activity is enhanced 1.5-fold in FD49 when compared with the wild type strain, whereas FPP synthase and squalene synthase specific activities were similar in both strains. Therefore the increase in the ergosterol level observed in FD49 could be specifically related to the enhancement of IPP isomerase specific activity, suggesting that IPP isomerase is a limiting step in the ergosterol pathway in yeast.

The IPP isomerase gene (17) was amplified by PCR and inserted in an expression vector under the control of

TABLE 6

Analysis of Free and Esterified Sterols in the Mutant Strain FD49 and in the Wild Type Strain as Function of Fenpropimorph Concentration[a]

	Ergosterol		Ergost-8-enol		Ignosterol	
Fenpropimorph[b]	Free	Ester	Free	Ester	Free	Ester
Wild type strain						
0	69	31	—[c]	—	—	—
0.066	100	0	34	66	—	—
0.66	100	0	15	85	19	81
Mutant strain FD49						
0	65	35	—	—	—	—
0.066	100	0	10	90	21	79
0.66	100	0	0	100	15	85

[a]The cells were grown for 48 h (complete medium) in the presence of different concentrations of fenpropimorph. The free sterols were separated from the esters as described in the Experimental Procedures section. The results are expressed for each sterol in % of the total sterols.
[b]Fenpropimorph concentration is expressed in μM.
[c]—, Not detected.

TABLE 7

Rate of Sterol Biosynthesis in the Wild Type Strain and in the Mutant Strain FD49

Substrate	FL100	FD49
[^{14}C-methyl]methionine[a]	0.34 ± 0.03 (3)	0.57 ± 0.03 (2)
[^{14}C]MVA[b]	0.20 ± 0.01 (3)	0.32 ± 0.01 (3)
[^{14}C]IPP[b]	0.14 ± 0.02 (5)	0.22 ± 0.01 (2)

[a]Cells were grown in minimum medium in the presence of radioactive methionine. Incorporation of radioactivity into nonsaponifiable lipids was determined. Results are expressed in μmoles per min per mg protein ± SD (number of independent experiments).
[b]*In vitro* transformation of labelled substrates into nonsaponifiable lipids. Results are expressed in nmoles squalene per min per mg protein ± SD (number of independent experiments). MVA, mevalonic acid; IPP, isopentenyl diphosphate.

TABLE 8

Enzyme Specific Activities in the Mutant Strain FD49 and in the Wild Type Strain[a]

	IPP[b] isomerase	FPP[b] synthase	Squalene[c] synthase
FL100	4.4 ± 0.7 (7)	5.0 ± 0.1 (3)	0.58 ± 0.02 (2)
FD49	6.4 ± 0.4 (3)	5.0 ± 0.1 (2)	0.57 ± 0.02 (2)

[a]Growth medium was complete medium.
[b]Specific activity is expressed as nmoles [^{14}C]isopentenyl diphosphate (IPP) incorporated per min per mg protein ± SD (number of independent experiments).
[c]Specific activity is expressed as nanomoles [^{14}C]farnesyl diphosphate (FPP) converted into squalene per min per mg protein ± SD (number of independent experiments).

GAL10-CYC1 inducible promoter (16). The transformed strain DD21 grown on galactose as carbon source showed a tenfold increase in IPP isomerase (23.3 nmol min^{-1} mg $prot.^{-1}$) specific activity when compared with glucose grown cells (2.4 nmol min^{-1} mg $prot.^{-1}$). In spite of this tenfold increase, no apparent increase in ergosterol level was noted (data not shown). In addition the strain DD21 did not show any increased resistance to fenpropimorph on galactose medium (data not shown). Thus, it can be concluded that the increase in IPP isomerase activity in FD49 is not, in itself, cause of both ergosterol enhancement and general resistance to inhibitors.

The fen1-1 *mutation suppresses the specific ergosterol requirement of yeast.* If one assumes that the toxic effect of the SBI is directly related to the starvation in "sparking" ergosterol, some mutations could by-pass this ergosterol requirement. We have shown that strains bearing a disrupted allele of sterol 14-reductase are unable to grow in aerobiosis unless they carry mutations such as *erg12-2* or *aux30* (27) allowing sterol uptake. In either case they are able to grow in the presence of a sparking ergosterol supplement (27). In order to check whether *fen1* allele relieves the specific ergosterol requirement, we constructed the recombinant strain, CM59, bearing the *fen1* allele in addition to a disrupted sterol 14-reductase allele.

Strain CM59 *(14str::TRP1, fen1)* was able to grow in aerobiosis, without the need of exogenous ergosterol (Fig. 1), unlike the parent strain defective in sterol 14-reductase. The nonsaponifiable lipid fraction of CM59 analyzed by GC contained ignosterol (70%), lanosterol (19%), squalene (10%) and zymosterol (1%), but ergosterol was not detected. These results show that ignosterol can efficiently replace ergosterol as a bulk membrane component and that the *fen1-1* mutation can overcome the specific ergosterol need of yeast cells.

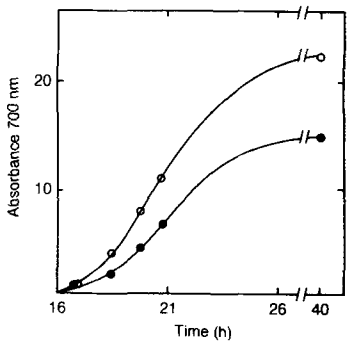

FIG. 1. Growth curves of FD49 *(fen1-1)*, and CM59 *(14str::TRP1, fen1-1)*. Cells were inoculated in complete medium at 28°C. The absorbance of the culture was measured after overnight growth; ○, FD49 *(fen1-1)*; ●, CM59 *(14str::TRP1, fen1-1)*.

DISCUSSION

The screening for fenpropimorph resistance allowed us to isolate a yeast mutant showing a pleiotropic phenotype, consisting of an enhancement of the ergosterol content and cross-resistance to numerous SBI.

The genetic analysis showed that this phenotype is linked to a unique recessive mutation, located on chromosome III, that we named *fen1-1*. The general resistance to SBI strongly suggests a mechanism of resistance linked to a common feature of the SBI, that is the generation of ergosterol starvation. The accumulation of the same intermediate sterols (ergost-8-enol and ignosterol) in the presence of fenpropimorph in *fen1-1* mutant and in the wild type strains excludes mechanisms of resistance such as loss of sensitivity of the target enzymes (28), diminution of drug concentration inside the cells related either to increased excretion of the fungicide (29) or reduced uptake of fungicide by the mutant strain (30), or a detoxication of products accumulated by fungicide treatment as it has been reported for azole-resistant mutants defective in sterol 5(6)-desaturation (31). We show here that there is no detoxication of the atypical sterols by esterification in FD49. Moreover the mutant strain remains sensitive to growth inhibitors independent of the sterol pathway (*e.g.*, cycloheximide, allyl alcohol, or copper) allowing one to exclude resistance due to a general loss of membrane permeability.

The increased ergosterol level in FD49, however, suggested another mechanism of resistance linked to the ergosterol overproduction. The 1.5-fold specific increase in IPP isomerase specific activity observed *in vitro* may by itself explain the ergosterol enhancement. However this hypothesis was ruled out since a high level of IPP isomerase in a wild type strain, obtained by gene overexpression, did not increase the amount of cellular ergosterol and the resistance to SBI.

An alternate explanation for the general resistance to SBI is that the *fen1-1* mutation exempts the cells from the need of the sparking ergosterol. This hypothesis could be tested by the construction of strains disrupted in sterol 14-reductase. Such strains require a sparking ergosterol amount for aerobic growth, but if in addition they carry the *fen1* allele, the need for ergosterol is no longer observed.

Dahl *et al.* (32) reported that a protein kinase, probably involved in the exit from the G1 phase, is stimulated by very low levels of ergosterol. The functional product of the *FEN1* gene could correspond to a protein linking ergosterol to cell proliferation, but since the *fen1* allele is recessive, it cannot correspond to the structural gene of this protein kinase. In fact a mutant gene encoding a constitutively-stimulated protein kinase would be dominant. This leads us to assume that the functional *FEN1* allele most likely encodes a negative transcriptional regulator of a gene, the product of which is involved in the control of the G1 phase exit; transcription of this gene could take place if ergosterol binds to the *FEN1* gene product. The *fen1* allele might encode a defective protein leading to constitutive expression of the hypothetical gene involved in G1 phase exit; in this case ergosterol would no longer be required. In addition, the *fen1* allele would lead to an increased flux in sterol biosynthesis suggesting that the protein encoded by *FEN1* is also implicated in the regulation of the ergosterol pathway.

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Effect of Lipid Content of Diet on Cholesterol Content and Cholesterogenic Enzymes of European Eel Liver

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The effect of dietary lipid levels on the levels of cholesterol and the activities of the major cholesterogenic enzymes of the liver has been studied in the European eel. An increase in hepatic total cholesterol was observed when the dietary lipid levels increased from 12 to 20%, while protein levels were maintained at 30%. This change paralleled an increase in mevalonate 5-pyrophosphate decarboxylase activity, while 3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate kinase and mevalonate 5-phosphate kinase were not affected by changes in diet composition. These results suggest that the decarboxylase may be a rate-limiting enzyme in cholesterogenesis in eel liver.
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Little is known about cholesterol metabolism in freshwater fish. In studies with northern pike (1) and carp (2), a small portion of radioactivity recovered in tissue lipids after injection of [^{14}C]acetate was associated with steryl esters. It also has been reported that the nutritional state of the animals did not affect the incorporation of [^{14}C]acetate into sterols by liver slices from European eel (3). More recently, it has been shown that the rate of cholesterol synthesis in trout hepatocytes is related to the glycogen content of the liver from which the hepatocytes were prepared (4).

Although the pathway of cholesterol synthesis in fish has not been examined in great detail, it can be assumed that the conventional mammalian route *via* 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is operative (5). In mammalian liver and other tissues, HMG-CoA reductase is the rate-limiting enzyme in cholesterogenesis (6). Changes in the activity of this enzyme correlate with changes in cholesterol synthesis in response to different experimental conditions (7,8). Nevertheless, the existence of secondary regulatory sites in cholesterol biosynthesis after HMG-CoA reductase has also been proposed (9–11). Results from our laboratory on the evolvement of chick mevalonate 5-pyrophosphate (MVAPP) decarboxylase during embryonic (12) and postnatal (13) stages, as well as the MVAPP decarboxylase changes in response to changing nutritional conditions (14,15), may suggest the importance of this enzyme in the regulation of cholesterol biosynthesis.

Until now, the enzymes responsible for cholesterogenesis in fish had not been characterized. In the present study we have investigated the effect of the lipid content of the diet on the main cholesterogenic enzymes in eel liver. Our results show that HMG-CoA reductase, mevalonate (MVA) kinase and mevalonate 5-phosphate (MVAP) kinase were not affected by changes in dietary lipid composition, while MVAPP decarboxylase activity was increased when dietary lipid content was increased from 12 to 20%. Thus, MVAPP decarboxylase activity may be related to the increases in hepatic cholesterol levels observed after such dietary manipulation.

MATERIALS AND METHODS

3-Hydroxy-3-methyl[3-^{14}C]glutaryl-CoA and [1-^{14}C]- and [2-^{3}H]mevalonic acid lactone were supplied by Amersham International (Amersham, United Kingdom). All other reagents were analytical grade. Fish oil was supplied by Afansa (Vigo, Spain).

Wild European eels *(Anguilla anguilla)* from the coastal marshes in southwest Spain were weight-selected and acclimated to fresh water for at least 3 wk by keeping them in 350-L fiber-glass tanks at a constant photoperiod (12 h light/12 h dark) prior to the experiments. Temperature was maintained at 25°C to obtain a faster growth (16). Aeration was continuously provided and water flow was maintained at about 1 L/min. Each experiment was comprised of 50 young fish in the fattening stage with an initial weight of about 50 g each.

Experimental diets were prepared as shown in Table 1 using three lipid levels (12, 16 and 20% per dry matter) and 30% of protein. The amount of herring meal used in all diets contained 4.91 g of lipid and 30 g of protein. Because of this, fish oil was added in the amounts necessary to reach the desired levels (7.5, 10.0 and 12.5 g).

TABLE 1

Composition of Experimental Diets (% of dry matter)

	Diets[a]		
	30/12	30/16	30/20
Herring meal protein	30.00	30.00	30.00
Herring meal oil	4.91	4.91	4.91
Herring meal others	9.87	9.87	9.87
Fish oil	2.59	5.09	7.59
Corn oil	4.50	6.00	7.50
Vitamin mixture[b]	2.00	2.00	2.00
Mineral mixture[c]	7.00	7.00	7.00
Sodium alginate	2.50	2.50	2.50
Cr_2O_3	0.50	0.50	0.50
Cooked starch	23.00	23.00	23.00
Cellulose	13.13	9.13	5.13

[a]30/12, 30% protein/12% lipid; 30/16, 30% protein/16% lipid; 30/20, 30% protein/20% lipid.
[b]Vitamin mixture (mg/100 g feed): calcium pantothenate, 6; thiamine, 2; riboflavin, 5; pyridoxine, 1.5; inositol, 40; biotin, 0.05; folic acid, 0.5; choline chloride, 120; nicotinic acid, 10; cyanocobalamin, 0.01; ascorbic acid, 40; vitamin A, 2; menadione, 1; vitamin E, 0.3; vitamin D, 0.4; cellulose, 1771.
[c]Mineral mixture (mg/100 g feed): $Ca(PO_4H_2)_2$. H_2O, 1500; $CaCO_3$, 1400; KH_2PO_4, 1000; KCl, 100; NaCl, 600; $MnSO_4 \cdot H_2O$, 35; $FeSO_4 \cdot 7H_2O$, 150; $MgSO_4$, 500; KI, 2; $CuSO_4 \cdot 5H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 25; $CoSO_4$, 3; Na_2MoO_4, 0.8; $Na_2SeO_3 \cdot H_2O$, 0.2; $Al_2(SO_4)_3 \cdot 18H_2O$, 2; cellulose, 1677.

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Abbreviations: CoA, coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; MVAP, mevalonate 5-phosphate; MVAPP, mevalonate 5-pyrophosphate.

Corn oil was added in the amounts necessary to reach a fish oil/corn oil ratio of 1.66 in all diets. Fish were fed twice a day by placing the diets in submerged baskets with free access to the fish for 15 min. Each diet was fed to two groups during the experimental period of 60 d. Each group consumed a similar amount of each diet. Weight gain was maximal in the animals fed the 30% protein/20% fat (30/20) diet.

At the end of the experimental period, the fish were weighed, killed, and the livers excised. The weighed livers were homogenized in 4 vol of 50 mM phosphate buffer (pH 7.4) containing 30 mM ethylenediaminetetraacetic acid, 250 mM NaCl and 1 mM dithiothreitol. The broken cell preparation was centrifuged at 5,000 × *g* for 15 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 15,000 × *g* for 15 min to sediment mitochondria. Microsomes were obtained by centrifugation of the 15,000 × *g* supernatant fraction for 60 min at 105,000 × *g*. All operations were done at 4°C.

HMG-CoA reductase activity was measured essentially as described by Shapiro *et al.* (17), with the modifications described by Alejandre *et al.* (18). Reductase activity was expressed as pmol of MVA synthesized per min per mg protein. MVA phosphorylation and decarboxylation was measured as previously described (11) using the 105,000 × *g* supernatant and [1-^{14}C]MVA as substrate. Total MVA kinase activity was measured by adding the MVAP and MVAPP formed during the reaction to the CO_2 formed. Similarly, MVAP kinase activity was measured by adding MVAPP and CO_2 formed. MVAPP decarboxylase activity was considered as the CO_2 formed. Specific activities were expressed as pmol per min per mg protein. Protein contents were determined by the method of Lowry *et al.* (19) using bovine serum albumin as standard.

Lipids were extracted with chloroform/methanol (2:1, vol/vol) as described by Folch *et al.* (20). Total and free cholesterol contents were determined by enzymatic/colorimetric methods using the "Test-Combination Cholesterol" or the "Test-Combination Free Cholesterol" kits from Boehringer Mannheim GmbH (Mannheim, Germany). The fatty acid composition of the different diets was determined according to Morrison and Smith (21) using gas chromatography as previously described (22). Methyl esters were analyzed using a Hewlett-Packard (Palo Alto, CA) 5880 A gas chromatograph equipped with a flame-ionization detector and a glass column (4 m × 2 mm) containing 12% SP 2340 on chromosorb WAW (100–120 mesh). The column temperature was kept at 180°C for 8 min and then raised by 5°C per min to a final temperature of 260°C. The nitrogen carrier gas flow rate was 30 mL/min. The column was standardized by injection of a standard mixture of fatty acid methyl esters, and unknown fatty acid methyl esters were identified by comparing their retention times with those of standards. Peak areas were determined by electronic integration and expressed as relative percentage of total fatty acids.

RESULTS

The effect of different experimental diets on the cholesterol content of eel liver was measured after 60 d of treatment. As can be seen in Table 2, feeding a 30% protein/20% fat (30/20) diet increased total hepatic cholesterol when compared with the 30/12 and 30/16 diets. This increase was mainly due to an increase in free cholesterol, a fraction that represented about 80–85% of liver cholesterol in all the experimental treatments.

In order to investigate the relationship between the changes in cholesterol content and the major regulatory enzymes in cholesterogenesis, we measured the effect of the diets on the specific activities of hepatic HMG-CoA reductase, MVA kinase, MVAP kinase and MVAPP decarboxylase. The results in Table 3 show that no significant differences were observed in HMG-CoA reductase specific activities upon any of the dietary manipulations. Similarly, MVA kinase and MVAP kinase did not change when the fat content of the diet was altered. Differences observed in $^{14}CO_2$ production from [1-^{14}C]MVA could be related only with MVAPP decarboxylase. Feeding the 30/20 diet produced a large increase in decarboxylase activity which was correlated with the total and free cholesterol content measured in the same tissue.

In order to correlate the changes in hepatic cholesterol content and enzyme activities with the different experimental diets, we analyzed the fatty acid compositions of these diets. Table 4 shows that no major differences were observed in the percentages of total saturated and unsaturated fatty acids. No significant differences were found in the levels of total polyunsaturated fatty acids in each diet, as well as in the percentages of ω3 and ω6 fatty acids. The saturated/unsaturated and saturated/polyunsaturated fatty acid ratios were essentially the same in the diets assayed. Similarly, no significant differences were observed in the ω3/ω6 ratio.

TABLE 2

Effect of Experimental Diets on Cholesterol Content (mg/g wet tissue) of Eel Liver[a]

	Diets[b]		
	30/12	30/16	30/20
Total cholesterol	3.02 ± 0.12	2.98 ± 0.07	4.62 ± 0.05[c]
Free cholesterol	2.48 ± 0.11	2.39 ± 0.13	3.89 ± 0.04[d]
Esterified cholesterol	0.54 ± 0.10	0.59 ± 0.14	0.73 ± 0.06[e]

[a]Results are expressed as means ± SEM of three determinations carried out with pools of five animals.
[b]30/12, 30% protein/12% lipid; 30/16, 30% protein/16% lipid; 30/20, 30% protein/20% lipid.
[c]$P < 0.0005$; [d]$P < 0.005$; [e]$P < 0.05$ with respect to 30/12 diet.

TABLE 3

Effect of Experimental Diets on Cholesterogenic Enzyme Activities in Eel Liver[a]

Specific activities (pmol/min/mg protein)	Diets[b]		
	30/12	30/16	30/20
HMG-CoA reductase	36.9 ± 2.2	35.5 ± 5.9	34.8 ± 2.2
MVA kinase	582.3 ± 18.3	638.3 ± 17.7	569.7 ± 5.7
MVAP kinase	199.2 ± 8.3	236.0 ± 8.0	201.6 ± 2.7
MVAPP decarboxylase	3.9 ± 0.3	6.7 ± 0.6[c]	12.1 ± 1.0[d]

[a]Results are expressed as means ± SEM of three determinations carried out with pools of five animals. HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonate; MVAP, MVA 5-phosphate; MVAPP, MVA 5-pyrophosphate.
[b]30/12, 30% protein/12% lipid; 30/16, 30% protein/16% lipid; 30/20, 30% protein/20% lipid.
[c]$P < 0.05$; [d]$P < 0.001$ with respect to 30/12 diet.

TABLE 4

Fatty Acid Composition of Experimental Diets (% of total fatty acids)[a]

Fatty acid	Diets[b]		
	30/12	30/16	30/20
14:0	3.0 ± 0.4	3.1 ± 0.2	3.3 ± 0.3
16:0	20.6 ± 1.7	19.5 ± 2.2	18.3 ± 1.9
16:1ω7	4.9 ± 0.6	5.9 ± 0.7	5.7 ± 0.8
18:0	2.9 ± 0.4	2.2 ± 0.3	2.6 ± 0.4
18:1ω9	21.2 ± 1.9	19.6 ± 1.7	21.4 ± 1.8
18:2ω6	17.2 ± 2.1	20.8 ± 2.3	20.3 ± 2.2
18:3ω3	2.9 ± 0.2	2.6 ± 0.3	2.6 ± 0.4
18:4ω3	2.0 ± 0.2	2.2 ± 0.3	2.3 ± 0.2
20:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
20:1ω9	6.1 ± 0.8	5.5 ± 0.6	5.4 ± 0.5
20:4ω6	1.3 ± 0.1	1.0 ± 0.2	1.3 ± 0.1
20:5ω3	8.1 ± 0.9	8.2 ± 0.9	8.0 ± 0.8
22:4ω6	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
22:5ω3	1.3 ± 0.2	1.2 ± 0.2	1.4 ± 0.2
22:6ω3	7.2 ± 0.6	7.0 ± 0.7	6.0 ± 0.8
Others	0.8 ± 0.1	0.5 ± 0.1	0.8 ± 0.1
Total saturated	26.8 ± 1.8	25.0 ± 2.2	24.4 ± 1.9
Total unsaturated	72.4 ± 3.2	74.4 ± 3.2	74.8 ± 3.2
Total polyunsaturated	40.3 ± 2.4	43.2 ± 2.6	42.3 ± 2.5
Total ω3	21.5 ± 1.1	21.2 ± 1.2	20.3 ± 1.2
Total ω6	18.8 ± 2.1	22.2 ± 2.3	22.0 ± 2.2
Saturated/unsaturated	0.37 ± 0.02	0.34 ± 0.03	0.33 ± 0.02
Saturated/polyunsaturated	0.66 ± 0.05	0.68 ± 0.06	0.58 ± 0.05
ω3/ω6	1.14 ± 0.14	0.96 ± 0.11	0.92 ± 0.10

[a]Results are expressed as means ± SEM of three determinations.
[b]30/12, 30% protein/12% lipid; 30/16, 30% protein/16% lipid; 30/20, 30% protein/20% lipid.

DISCUSSION

In a previous study, we have shown that the dietary protein content (35–55%) did not affect the triglyceride and phospholipid content of eel liver when the lipid level of the diet was maintained at about 12% (23). However, feeding a herring meal 55% diet increased the total cholesterol content in eel liver, while feeding a white fish meal 55% diet did not cause significant changes in hepatic cholesterol (24). Given that the ω3/ω6 fatty acid ratio was manifestly lower in herring meal 55% diet, differences in the hepatic cholesterol content may have been related to the fatty acid composition and not the protein content of the diet. In the present study, we observed a substantial increase in hepatic cholesterol content when the lipid level of the diets was increased from 12 to 20%, while the protein level was maintained at 30%.

It is generally accepted that the relationship between cholesterol content and the activities of cholesterogenic enzymes can be used as a measure to assess the importance of a given enzyme in cholesterol metabolism. However, no significant differences were observed in HMG-CoA reductase activity after any of the dietary treatments. By contrast, hepatic MVAPP decarboxylase drastically increased with increasing lipid content of the diet parallelling the increase in liver cholesterol content. This suggests that decarboxylase may be the rate-limiting enzyme in cholesterogenesis in eel liver, as it has been

reported in other animal species under various physiological conditions (9-15). Since the fatty acid composition was essentially the same in all the diets assayed, the increased cholesterol content and increased MVAPP decarboxylase activity appear to be related to the increased fat content of these diets.

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Parameters Influencing Cholesterol Oxidation

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The purpose of this study was to investigate the effects of temperature, oxidation time, presence of water, pH, type of buffer and form of substrate used on cholesterol oxidation. Microcrystalline cholesterol films, both solid and melted, and aqueous suspensions of film fragments were used as substrates. Use of dispersing agents was avoided. Quantitative analysis of the unaltered substrate and the products of its autoxidation was carried out by gas chromatography over the course of oxidation. Solid cholesterol films were found to be resistant to autoxidation in the dry state. However, when heated at 125°C, a sudden increase in oxidation rate occurred at a point coinciding with the visible melting followed by a plateau of the oxidation rate. All of the autoxidation products formed underwent further decomposition. Film fragments of cholesterol oxidized at a faster rate in aqueous suspensions than when oxidized in the dry state. In aqueous suspensions, the differences in the resistance of cholesterol to oxidation were not significant within the pH range 6.0–7.4, except for the early stages of oxidation. The 7-ketocholesterol/7-hydroxycholesterol ratio dropped significantly with increasing pH. However, at all pH levels tested, this ratio remained relatively constant during the 6 h of heating. While the 7β-hydroxycholesterol/7α-hydroxycholesterol ratio was not affected by pH in the range of 6.0–7.4, at pH 7.4 a high preference was observed for the cholesterol β-epoxide over its α-isomer. The β/α-epoxide ratio decreased with time of heating and with decreasing pH. The data show that the physical state of the substrate exerts a major influence on the oxidative behavior of cholesterol.
Lipids 28, 917–922 (1993).

The products of cholesterol oxidation and the mechanisms for their formation have been reviewed (1–3). In general, the epimeric 3β-hydroxycholest-5-en-7α- and 7β-hydroperoxides (7α- and 7β-hydroperoxides) are recognized as the initial products, with the 7β-hydroperoxides being produced more abundantly than the α-isomers (1,4–6). Thermal decomposition of the hydroperoxides gives rise to the isomeric cholest-5-ene-3β,7α-diol(7α-hydroxycholesterol) and cholest-5-ene-3β,7β-diol(7β-hydroxycholesterol), the 5,6α- and the 5,6β-epoxy-5α-cholestan-3β-ols (cholesterol α-epoxide and cholesterol β-epoxide) and 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), with the latter being a major product.

Studies on cholesterol oxidation that have been reported differed with regard to the form of the substrate used, with sample preparation, with oxidants and with the methods of analysis. Only few studies have been reported in which the oxidizing systems were examined over the entire course of the autoxidation process. Consequently results varied, qualitatively and quantitatively, and the effects of various treatment conditions on cholesterol oxidation were often ill-defined. Oxidation products were also different when colloidal dispersions of cholesterol were used rather than when solid cholesterol was oxidized, and in suspensions dispersed with sodium stearate rather than when other dispersing agents were used (1,4,7–9). The dispersing agent itself can influence the course of oxidation or the profile of oxidation products.

The purpose of the present work was to study the effects of temperature, oxidation time, presence of water, pH and type of buffer on oxidizing cholesterol in different physical states (dried film, melted form, aqueous suspensions of film fragments) without the interference of dispersing agents.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol, *tris*(hydroxymethyl)aminomethane (Tris) and triethanolamine (TEA) were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol contained less than 0.9% contaminants including 7-ketocholesterol, 7β-hydroxycholesterol, cholesterol α- and β-epoxides and cholesta-3,5-dien-7-one. Other steroid standards [7-ketocholesterol, 7-hydroxycholesterols, cholesterol epoxides, 20- and 25-hydroxycholesterols, 5α-cholestane-3β,5,6β-triol (3β,5α,6β-triol), cholesta-3,5-diene, cholesta-3,5-dien-7-one, etc.] were from Steraloids Inc. (Wilton, NH). *N,O-bis*(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). Solvents including water were high-performance liquid chromatography (HPLC) grade from various sources.

Sample treatment. Solutions of cholesterol in chloroform (1 mg in 100 μL) were pipetted into vials (15 mm diameter, 45 mm long), and the solvent evaporated on a rotary evaporator. This resulted in the formation of a thin film on the bottom of each vial. Under the microscope, these preparations appeared as thin films of needle-shaped microcrystals, 10–40 μm in length. Some more dense regions appeared to consist of several such layers while other regions appeared amorphous. For studies with dry cholesterol, the vials containing 1 mg of the cholesterol film were placed in an oven at preset temperature and heated for specified periods of time. For the aqueous samples, 1 mL of water or buffer solution was added to test tubes (16 mm diameter, 125 mm long), containing 1 mg of cholesterol film, prepared as described above, and sonicated (Branson Ultrasonic Co., Danbury, CT) for 1 min to detach and break the cholesterol film into suspended small fragments. Under the microscope these fragments appeared as multilamellar structures suspended throughout the aqueous medium. Preliminary tests by gas chromatography (GC) showed that sonication did not generate detectable amounts of cholesterol oxides. The aqueous samples were incubated with shaking at 75°C for up to 6 d or at 37°C up to 30 d.

Cholesterol analysis. Analyses of cholesterol and its oxidation products were carried out as described previously (10). Dry samples were silylated with 0.5 mL BSTFA plus 1% TMCS at 80°C for 1 h. For aqueous samples,

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Abbreviations: BSTFA, *N,O-bis*(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; HPLC, high-performance liquid chromatography; TEA, triethanolamine; TMCS, trimethylchlorosilane; Tris, *tris*(hydroxymethyl)aminoethane.

cholesterol and its oxidation products were extracted once with 3.5 mL of $CHCl_3$/MeOH solvent mixture (2:1, vol/vol). The extraction yields (relative to the internal standard) of cholesterol, 7-hydroxycholesterols, 5,6-epoxides, 7-ketocholesterol and 3β,5α,6β-triol were 98.3% (SD = 3.4%), 99.3% (SD = 4.3%), 105.5% (SD = 14.7%), 95.8% (SD = 5.0%) and 99.0% (SD = 4.2%), respectively. The extract was dried under a nitrogen stream and silylated. The silylated samples were analyzed by GC (Varian 3700; Palo Alto, CA) using an Ultra-1 capillary column (50 m × 0.2 mm × 0.33 μm, Hewlett-Packard, Avondale, PA). GC parameters were as follows: sample size, 0.2 μL; concentration, 0.5% (wt/vol); split ratio, 30:1; carrier gas flow rate, 0.3 mL He/min at 305°C. Oven temperature was programmed from 105 to 305°C at 10°C/min. 3β-Acetoxy-5α-androstan-17-one was used as an internal standard. GC peaks were identified by their retention times and by comparison of their mass spectra with those of authentic compounds. A Hewlett-Packard Model 5985 B gas chromatoaph/mass spectrometer system was used.

RESULTS AND DISCUSSION

Effect of temperature. In general, solid cholesterol showed high resistance to oxidation. At 110 and 120°C, where cholesterol remained solid, less than 10% was oxidized after 80 h of heating (Fig. 1). At 125°C, a temperature approximately 20°C below the melting point, cholesterol remained in the solid state for 20 h during which oxidation was slow. Beyond this point, melting became visible and a sudden increase in oxidation rate occurred. The melting of heated cholesterol below its melting point probably reflects the effect of trace amounts of oxidation products formed on the melting point. After approximately 40 h of heating, the sample was completely melted and the rate of oxidation decreased. It is not clear why such a decrease in the oxidation rate occurred, although the accumulation of oxidation products may have an inhibitory effect on the oxidation rate (1,5). The same phenomenon was also observed at 130°C and to a lesser degree at 135°C. At higher temperatures, *e.g.*, 150 and 180°C, cholesterol was in the liquid state from the beginning of heating, and more than 80% of the initial cholesterol disappeared within 1 h of heating. The remaining cholesterol levels after 30 min at 150°C and after 20 min and 40 min at 180°C were 24.4, 28.3 and 16.4%, respectively.

The oxidation products that were detected in the present study were those known to result from cholesterol oxidation: for example, 7-ketocholesterol, and 7α- and 7β-hydroxycholesterols from 7-derivatization; cholesterol α- and β-epoxides and 3β, 5α, 6β- triol from epoxidation; 20- and 25-hydroxycholesterol from side-chain derivatizations; and cholesta-3,5-diene, cholesta-3,5-dien-7-one and several other ketones from elimination reactions. Other products, *e.g.*, volatiles (1), relatively high molecular weight compounds (Vajdi, M., and Nawar, W.W., unpublished data) and other minor oxides, may have also been formed but could not be detected by the methods used in this study. Only traces of side-chain derivatives, *i.e.*, 20- and 25-hyoxycholesterols, and triols were detected in the dry-state oxidation.

Figure 2 shows the relationship between heating time and amounts of cholesterol decomposition products formed. The top line represents the total amounts of

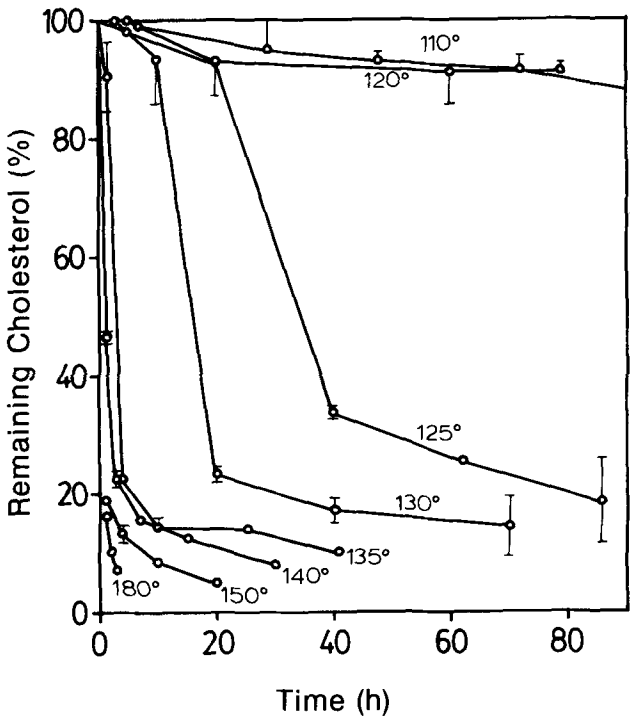

FIG. 1. Effect of temperature (average of two determinations) on the oxidation of dry cholesterol films, 1 mg each, prepared by placing chloroform solutions of cholesterol into vials (15 mm diameter, 45 mm long) and evaporating the solvent. Samples were heated in air in an oven at preset temperatures. Remaining unaltered cholesterol was determined by gas chromatography and is expressed as percent of initial substrate.

altered cholesterol. The bottom line represents that portion of the altered cholesterol which was observed as specific products, *i.e.*, 7-ketocholesterol, the 7-hydroxycholesterols, the cholesterol epoxides, cholesta-3,5-diene and cholesta-3,5-dien-7-one. The region d, which is the difference between total altered cholesterol and the sum of the 7-derivatives, epoxides and elimination products, represents other compounds not detected and measured in this study. Polymers and/or other compounds of relatively high molecular weight may constitute a major portion of such compounds. When cholesterol heated for 1 h at 180°C was subjected to size exclusion chromatography, a fraction amounting to 21.1% of the initial cholesterol represented material of higher molecular weight than the substrate (Vajdi, M. and Nawar, W.W., unpublished data).

Figure 3 illustrates the relationship between heating time and amounts of 7-ketocholesterol formed, one of the major products detected in the early stage of cholesterol oxidation. Obviously, 7-ketocholesterol, and other products as well, undergo further decomposition during heating; the amount of 7-ketocholesterol at any given time represents the net balance between formation and decomposition. As expected, the higher the temperature, the faster the production and decomposition of the oxides. The amounts of 7-ketocholesterol produced in the course of heating at different temperatures (Fig. 3) are consistent with the oxidative behavior of cholesterol discussed

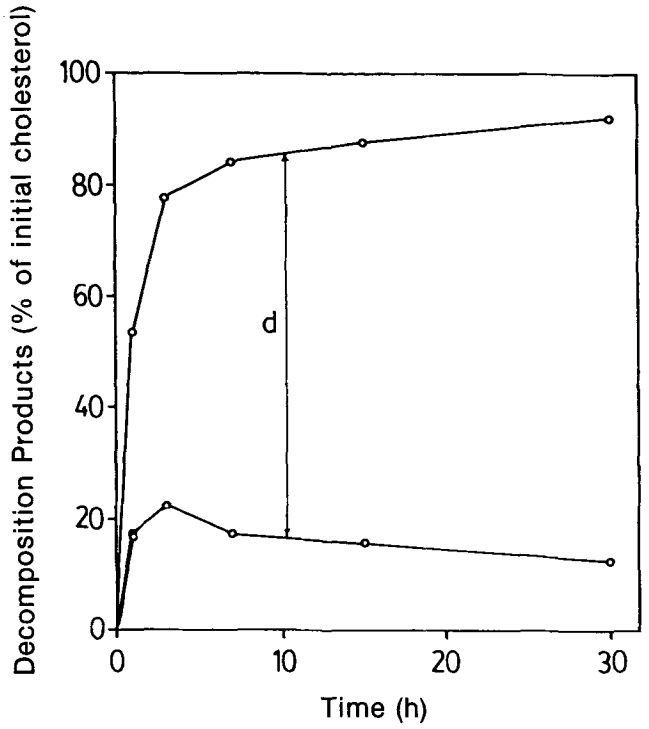

FIG. 2. Production of cholesterol decomposition products in dry cholesterol films heated in air at 140°C (average of two determinations). Samples, 1 mg each, were prepared by placing chloroform solutions of cholesterol into vials, 15 mm diameter × 45 mm long, and evaporating the solvent. Remaining unaltered cholesterol and decomposition products were determined by gas chromatography; ○—○, total amount of altered cholesterol; ●—●, sum of oxidation products detected; region d, sum of undetected products.

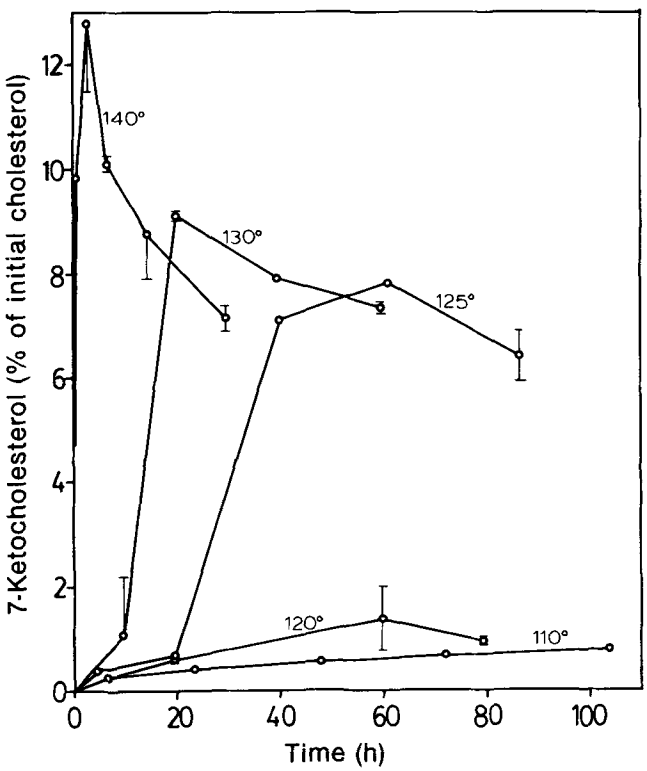

FIG. 3. Effect of temperature on the production of 7-ketocholesterol in dry cholesterol films (average of two determinations). Samples, 1 mg each, were prepared by placing chloroform solutions of cholesterol into vials, 15 mm diameter × 45 mm long, and evaporating the solvent. Samples were heated in air in an oven at preset temperatures, and 7-ketocholesterol was monitored by gas chromatography of their silyl derivatives.

previously (Fig. 1). At 110 and 120°C, the rates of 7-ketocholesterol production were slow and not too different from each other. However, 7-ketocholesterol formation was markedly increased at 125°C when cholesterol melting was seen.

To illustrate the effect of heating on the products of cholesterol oxidation, and the effect of these on cholesterol oxidation, several heating experiments were carried out with pure compounds. When dry film of 7-ketocholesterol, prepared in the same manner as described above for cholesterol, was heated alone at 150°C, after 10 h of heating 83% of the initial amount remained unchanged. However when mixed with pure cholesterol before heating, 64% of the initial 7-ketocholesterol and 33% of the initial cholesterol remained. When a mixture containing equal amounts, 1 mg each, of 7-ketocholesterol, 7β-hydroxycholesterol, cholesterol β-epoxide and 3β, 5α,5α,6β-tril were similarly heated, the amounts found after 10 h heating were 53, 6, 5 and 16%, respectively. These results confirm the decomposition of 7-ketocholesterol upon heating and the difference in stability of the various oxidation products when heated while exposed to air. Not only do these products influence the oxidation of cholesterol, but also the oxidation of each other.

Figure 4 shows formation of the 7-hydroxycholesterols in the dry system during autoxidation for 30 h at 140°C. While most studies show approximately equal yields of the 7α- and the 7β-hydroxycholesterol isomers (4,5,11), the reported β/α ratios of the epoxides varied widely

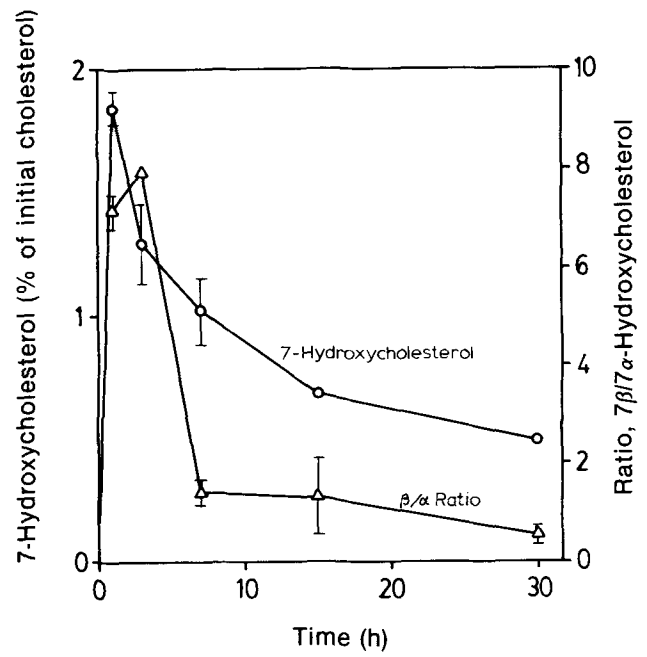

FIG. 4. Effect of heating time on the levels of 7-hydroxycholesterols in dry film fragments at 140°C (average of two determinations). Samples, 1 mg each, were prepared by placing chloroform solutions of cholesterol into vials, 15 mm diameter × 45 mm long, and evaporating the solvent. The 7α- and 7β-hydroxycholesterols were monitored by gas chromatography of their silyl derivatives.

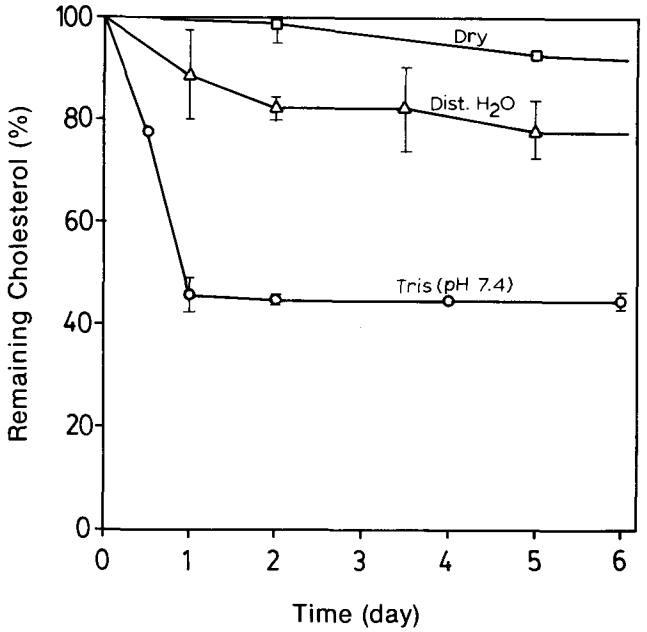

FIG. 5. Effect of medium on cholesterol oxidation at 75°C (average of three determinations). Samples were prepared by adding 1 mL of water or buffer to test tubes, 16 mm diameter × 125 mm long, contianing 1 mg of cholesterol film and sonicating for 1 min. Remaining unaltered cholesterol was determined by gas chromatography of its silyl derivative and is expressed as percent of initial substrate. Tris; *tris*(hydroxymethyl)aminoethane; Dist., distilled.

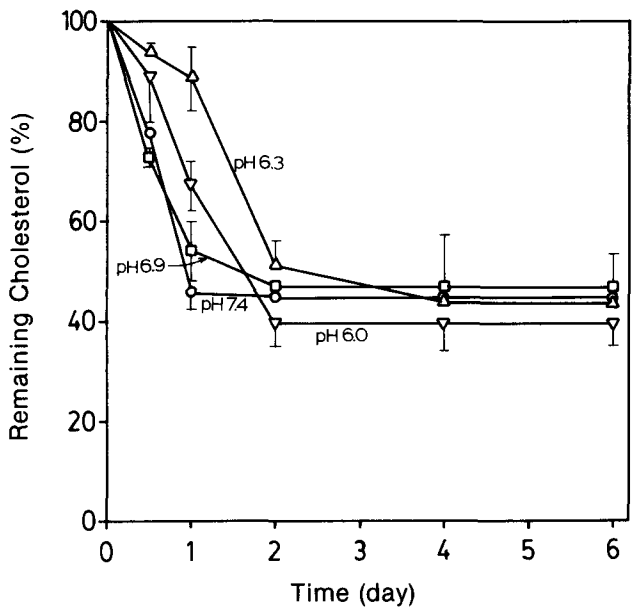

FIG. 6. Effect of pH on cholesterol oxidation in film fragments suspended in tris buffer, 75°C (average of three determinations). Samples were prepared by adding 1 mL of buffer solution to test tubes, 16 mm diameter × 125 mm long, containing 1 mg of cholesterol film and sonicating for 1 min. Remaining unaltered cholesterol was determined by gas chromatography of its silyl derivative and expressed as percent of initial substrate.

depending on conditions (4,12-14). In our work with dry films, the level of 7-hydroxycholesterols reached 1.8% of the initial amount of cholesterol after 3 h heating, then decreased to 0.5% after 30 h (Fig. 4). The β/α-hydroxycholesterol ratio reached a maximum of 8 at the early stage of heating then dropped rapidly and leveled at a ratio of approximately 1 as has been reported for studies with colloidal dispersions. Formation of the 5,6-epoxides was similar (data not shown). Their level reached about 7% of initial cholesterol after 3 h then decreased to 3% after 30 h of heating. The β/α ratio of the epoxides was 1.3 after 3 h, then dropped to a relatively constant ratio of around 0.3 after 6 h. This decrease in β/α ratios during heating probably reflects a faster degradation of the β-epoxides than of their α-isomers. Below 130°C, the β/α-epoxide ratio did not exceed 1.0 (data not shown),. At lower temperature, the overall rate of formation was reduced, but degradation of β-epoxide may not be as sensitive to temperature change as degradation of the α-form.

Effect of water. Cholesterol oxidation was faster in the presence of water than in the dry state, and faster in Tris buffer (10 mM) than in distilled water (Fig. 5). Several workers have reported that in aqueous dispersion the oxidation of cholesterol occurred in the early stage of incubation with no further change after 70-75% substrate had been consumed. Smith *et al.* (7) defined this phenomenon as the apparent product inhibition of the cholesterol oxidation reaction, while Maerker (3) suggested that this plateau may be caused by changes in micelle structure due to the presence of the oxidation products. In the present study, such a plateau was also observed. The phenomenon, however, may be due to factors other than those in aqueous dispersions. Since dispersing agents were not used, cholesterol existed not in micellar structure but as film fragments suspended in the aqueous medium. Cholesterol oxidation is likely to occur on the surface of these fragments (15) whereas their inside may remain inert causing the plateau. Any treatment which influences the structure of the fragments may thus have a significant effect on the oxidation in the latter stages.

The profiles of cholesterol oxidation products formed at 75°C in the absence and presence of water were compared (data not shown). Because of the slow oxidation at 75°C, the dry cholesterol film did not accumulate high amounts of oxidation products even after 40 d, but a number of products were detected. In contrast, in the presence of water the products were relatively few, *i.e.*, the 7-derivatives and the epoxides were the only major products detected. The levels of detectable oxidation products were much higher in the presence of water than those observed in the dry state. Therefore, in aqueous systems, 7-peroxidation and 5,6-epoxidation showed greater effects on cholesterol decomposition than in the dry state.

Effect of pH and buffer. Significant differences in cholesterol stability were observed within the pH range of 6.0-7.4, but only in the early stages of incubation (Fig. 6). After one day in 10 mM Tris-HCl buffer at 75°C, the remaining unaltered cholesterol amounted to 90% of the initial cholesterol at pH 6.3, 55% at pH 6.9 and 43% at pH 7.4. Between the second and sixth day, the remaining cholesterol was about the same for all pH values showing a plateau at 40-50%.

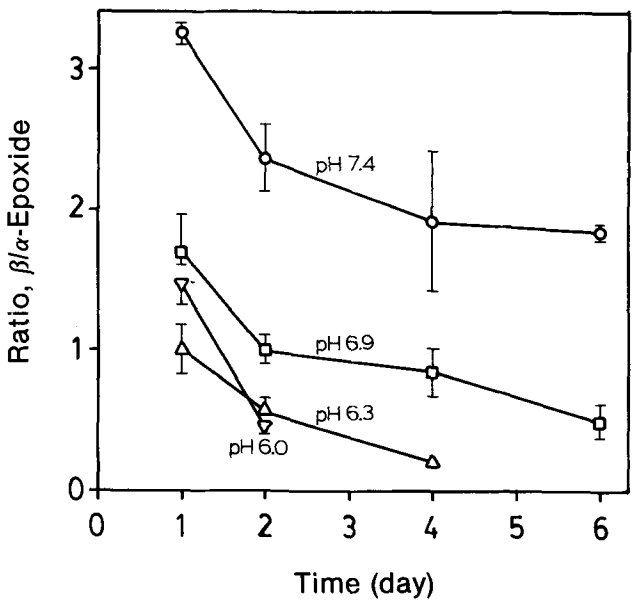

FIG. 7. Effect of pH on β/α-epoxide ratio in cholesterol film fragemts suspended in tris buffer, 75°C (average of three determinations). Samples were prepared by adding 1 mL of buffer solution to test tubes, 16 mm diameter × 45 mm long, containing 1 mg of cholesterol film and sonicating for 1 min. The α- and β-epoxycholesterols were monitored by gas chromatography of their silyl derivatives.

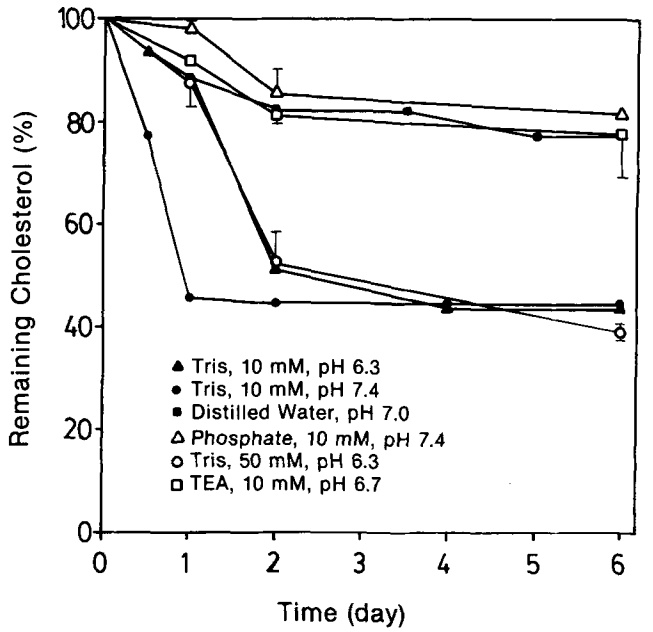

FIG. 8. Effect of different buffer systems on cholesterol oxidation in aqueous suspension of film fragments at 75°C (average of three determinations). Samples were prepared by adding 1 mL of water or buffer solution to test tubes, 16 mm × 45 mm long, containing 1 mg of cholesterol film and sonicating for 1 min. Remaining unaltered cholesterol was determined by gas chromatography of its silyl derivative and expressed as percent of initial substrate. TEA, triethanolamine. See Figure 5 for other abbreviation.

Ratios of 7-keto/7-hydroxycholesterol have been reported to be around 2 in aqueous media (6,5,10,13). In the present work the 7-keto/7-hydroxycholesterol ratio was approximately 7 at pH 6 and dropped with increasing pH reaching about 3 at pH 7.4. This trend was expected. Dehydration of the 7-hydroperoxide to yield 7-ketocholesterol occurs readily at lower pH, whereas the latter is particularly sensitive to alkaline conditions (3,16–18). At all pH levels tested, the 7-ketocholesterol/7-hydroxycholesterol ratios remained relatively constant during the 6-h heating period.

The stereoselectivity in 7-hydroxylation was not affected by pH in the range of 6.0–7.4, but as shown in Figure 7 higher β/α-epoxide ratios were observed at pH 7.4. Maerker and Bunick (4) found no significant change in the β/α-epoxide ratio 3–5 during the oxidation of cholesterol in aqueous dispersions at pH 8 and 12. However at pH 5.5, large changes in the α/β ratio were observed, and the β-epoxide was more labile than its α-isomer by a factor of 2.5.

Figure 7 shows that at all pH levels tested, the β/α-epoxide ratios were highest after one day of heating (the point where the total epoxide level reached a maximum) and then decreased with time of heating. It is also clear that the β/α-epoxide ratios were markedly decreased as the pH was reduced, reflecting the instability of the β-isomer in acidic media (4).

The effects of various buffer systems, 10 mM tris-HCl pH 6.0–7.4, 50 mM tris-HCl pH 6.3, 10 mM potassium phospshate pH 7.4, and 10 mM triethanolamine (TEA) HCl-NaOH pH 6.7, on cholesterol oxidation are shown in Figure 8. Phosphate and TEA buffers did not show any difference from the distilled water control, while tris buffer showed a destructive effect within the pH range of 6.0–7.4. The concentration of tris buffer (10 mM and 50 mM) did not influence cholesterol oxidation. Tsai and Smith (19) reported that the phosphate group and quaternary amine showed no effect on the oxidation of methyl linoleate while protonated primary amines accelerated lipid oxidation. In this study, TEA buffer, which has a quaternary amine and phosphate buffer, did not show any difference from the distilled water control while tris buffer, which has a protonated primary amine, accelerated cholesterol oxidation. The effect of such ionic species on free radical reactions remains unclear. Since the pH of tris buffer was adjusted with HCl, there was a possibility of a higher level of iron contamination than in other media. This possibility, however, is unlikely in view of the fact that the lower pH buffers, which would provide more Fe contamination, showed less cholesterol oxidation at the early stage of incubation. Also, increasing concentration of the tris buffer did not show a significant effect.

Distilled water, phosphate and TEA buffer produced similar product profiles and β/α ratios of epoxides and 7-hydroxycholesterols. Only in tris buffer, the β/α-epoxide ratio was higher than 1, and hydroxylated side-chain products, *e.g.*, 20-hydroxycholesterol and 25-hydroxycholesterol, were observed.

At 37°C, the variation among observations was very high (SD = 0.17–22%, average SD = 8%). The difference between the effects of tris and phosphate buffers at pH 7.4 was not as pronounced as that observed at 75°C. As

in the case of tris buffer in the early stage of incubation at 75°C (Fig. 6), cholesterol was more stable at pH 6.3 than at pH 7.4 in phosphate buffer at 37°C.

The experimental work detailed above was designed to provide basic information as part of an ongoing study on oxidation interactions of cholesterol with other components in more complex systems. For example, the approach used here allows the study of cholesterol/phospholipid co-oxidation without interference from dispersing agents. Such interactions will be discussed in a later report.

Cholesterol oxidation is system dependent. The products of cholesterol oxidation and their relative amounts vary depending on temperature, oxidation time, presence of water, pH, type of buffer and form of substrate. The effects of these parameters on the rate and products of oxidation appear to be largely manifested through their effects on the physical state of the substrate.

ACKNOWLEDGMENTS

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Cholesterol and 25-Hydroxycholesterol Retention in Specimens of Liver and Aorta Prepared for Electron Microscopy. I. Standard Fixation Methods and Metabolism of the Labeled Sterols

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In the present work, several preparatory procedures commonly used for electron microscopy (EM) were evaluated as to their ability to preserve cholesterol (CHO) and CHO derivatives in tissue. We also determined in several rat tissues to what extent the sterols used as tracers are metabolized. Sprague-Dawley rats were injected intraperitoneally with [1α,2α(n)-^{3}H]cholesterol ([^{3}H]CHO) and 25-hydroxy-[26,27-^{3}H]cholesterol ([^{3}H]25-OH-CHO). Lipids of the liver, aorta and brain were extracted one and five days after injection, and the distribution of the labeled lipids was followed by thin-layer chromatography. When labeled CHO was injected as tracer, most of the radioactivity remained associated with the CHO fraction. When 25-hydroxycholesterol (25-OH-CHO) was used, we found that it was mostly metabolized to yield more polar compounds. Our results show that the loss of CHO and CHO derivatives from tissues depends not only on the preparatory procedure used for EM, but also on the type of tissue studied. ***Lipids 28,*** **923–928 (1993).**

Lipids usually constitute about half of the membrane mass, and in most mammalian plasma membranes cholesterol (CHO) is present at an about equimolar ratio with phospholipids. Changes in CHO content affect membrane fluidity, permeability, electrical resistance, integral functions and hydration properties (1).

Conventional procedures used for the preparation of biological specimens for electron microscopy (EM) involve extensive loss of lipids from the specimens. Several approaches have been proposed to minimize this loss (2). Most previous studies have mainly been concerned with the fate of total lipids, and little information is available on how EM preparatory procedures affect the CHO content of plasma membranes (2). The retention of CHO as cell membrane component is desirable both to preserve membrane structural integrity and for the ultrastructural localization of these components.

Controversy also exists in regard to the types of lipid losses that occur during conventional EM preparatory procedures (2–4), and differences exist between the type of lipid losses that occur after fixation with aldehydes as compared to OsO_4, the two fixatives most commonly used. Whereas aldehydes do not prevent lipid loss in most tissues, there is evidence that OsO_4 may minimize the loss of some types of lipids (2). It is also not clear how CHO metabolites are affected.

In the present study we determined to what extent various labeled tracers are metabolized, and we followed the loss of the labeled lipids during the fixation procedures. The radioactive components present in the tissues before fixation were extracted and analyzed. We also determined the extent to which CHO and 25-hydroxycholesterol (25-OH-CHO) were lost from rat aorta and liver in the course of various standard procedures used for the preparation of animal tissues for conventional EM and CHO cytochemistry.

MATERIALS AND METHODS

Animals and tracers. Sprague-Dawley rats weighing 150–200 g were used. Animals were injected intraperitoneally (i.p.) with 1 mCi of [^{3}H]CHO, 40–60 Ci/mmol (Amersham International, Amersham, England), or [^{3}H]25-OH-CHO, 75–87 Ci/mmol (New England Nuclear, Boston, MA) 1 and 5 d prior to being sacrificed. Both products were prepared for injection by the method described by Baranowsky *et al.* (5). After evaporation of the toluene from the original vial containing the tracer, the labeled sterol was redissolved in 0.5 mL of ethanol. One mL of saline was added and most of the ethanol was evaporated by a stream of nitrogen. The resulting sterol suspension was injected i.p..

The optimum sampling time for fixation and for thin-layer chromatography (TLC) of the lipid extracts was determined by measuring the specific activity [disintegrations per minute (dpm)/g] in aortic tissue of rats 1, 2, 5, 7 and 13 d after i.p. injection of 0.125 Ci of [^{3}H]CHO or [^{3}H]25-OH-CHO per 100 g of animal weight. Maximum activity (dpm/g) was found at 5–7 d when [^{3}H]CHO was injected, and at 5 d when the tracer was [^{3}H]25-OH-CHO (Table 1). A sampling time of 5 d was therefore used for the present study. An additional sample was taken on the first day for TLC analysis to document the metabolic changes with time.

Rats were kept under pentobarbital anesthesia when removing tissue. After cannulating the aorta, saline was

TABLE 1

Radioactivity Found in Aortic Tissue 1, 2, 5, 7 and 13 Days After i.p. Injection with [^{3}H]CHO or [^{3}H]25-OH-CHO[a]

Days after i.p. injection	Tracer injected: [^{3}H]CHO dpm/g	Tracer injected: [^{3}H]25-OH-CHO dpm/g
1	4 757	8 646
2	6 945	15 233
5	11 118	29 335
7	13 122	10 229
13	6 389	5 408

[a]For details, see Materials and Methods Abbreviations: i.p., intraperitoneal; dpm, disintegrations, per minute; [^{3}H]CHO, [1α,2α(n)-^{3}H]cholesterol; [^{3}H]25-OH-CHO, 25-hydroxy[26,27-^{3}H]cholesterol.

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Abbreviations: CHO, cholesterol; [^{3}H]CHO, [1,α2α(n)-^{3}H]cholesterol; EM, electron microscopy; F, formaldehyde; G, glutaraldehyde; 25-OH-CHO, 25-hydroxycholesterol; [^{3}H]25-OH-CHO, 25-hydroxy-[26,27-^{3}H]cholesterol; dpm, disintegrations per minute; i.p., intraperitoneal; PB, 0.1 M phosphate buffer (pH 7.3, 2 mM $MgCl_2$); TA, tannic acid; TLC, thin-layer chromatography.

infused for one min at 100 mm Hg pressure. Immediately thereafter, aliquots of tissue from aorta and liver were taken for each of the fixation procedures. Samples of aorta, liver and brain were also taken to measure radioactivity and to extract lipids.

Lipid extraction and chromatography. Liver homogenates (2 g liver and 5 mL saline) and aortas were extracted with chloroform/methanol (2:1, vol/vol) by the Folch method (6). After evaporation of the chloroform phase, lipids were separated by TLC on a Kieselgel 60 plate (Merck, Darmstadt, Germany) with two developments in *n*-heptane/ethyl acetate (1:1, vol/vol). On the same plate and in parallel chromatograms, the following standards were used: CHO linoleate (Sigma, St. Louis, MO); CHO (Sigma); 5-cholestene-3β, 25-diol (Steraloids, Wilton, NH) and cholestane-3β, 5α, 6β-triol (Steraloids). The standards were made visible with iodine vapor, and the chromatograms were divided into nine areas, one containing the origin and four others containing the areas corresponding to the standards. The scraped areas were extracted with chloroform/methanol (2:1, vol/vol) and radioactivity logs counted after evaporation of the solvent.

Counting techniques. Radioactivity of the residual liquids of the individual steps of the fixative procedures was measured. As the chemical composition of the different samples varied greatly, the counting efficiency of the samples was determined using an internal standardization method, and the counts were converted into dpm. Special care was required to measure the samples containing osmium because of especially low counting efficiencies. The original dpm of the tissues to be fixed was determined by dissolving aliquots of the same type of tissue in Nuclear Chicago Solubilizer (Chicago, IL) and counting as described before. A toluene/Triton X-100 liquid scintillator (Scharlau, Barcelona, Spain) and LKB 1219 Rackbeta counter (LKB, Turku, Finland) were used. Results are the mean of three experiments; three animals were injected with each tracer, and two samples of tissue per animal were fixed independently in each of the fixative procedures studied.

Preparation of tissues for EM by Procedure G + Os. Aliquots of tissue from aorta and liver (1 mm^3) were immersed (7) in 1% glutaraldehyde (G) in 0.1 M phosphate buffer (PB) (pH 7.3, 2 mM $MgCl_2$) for 24 h at room temperature in the dark. Effective osmolarity of the fixative solution (300 mOsm) was determined as described (8,9). Purity of G (Merck) was determined by measuring the relative values of ultraviolet absorbance between 210 and 310 nm (8,10,11). The ultraviolet absorbance spectra showed a peak only at 280 nm. After a brief washing in PB, the tissues were postfixed with 2% OsO_4 containing 1.8% potassium ferrocyanide in PB for 2 h in the dark at room temperature (296 K).

Samples were dehydrated by 5-min immersions into ethanol (60, 80 and 95%)/water (vol/vol), and by two 5-min immersions into 100% ethanol; all immersions were at 273 K.

Epon 812 (Polysciences, Warrington, PA) consisted of 21 mL Poly/Bed 812, 13 mL dodecenyl succinic anhydride, 11 mL nadic methyl anhydride and 0.7 mL DMP-30, which was added just before use. Samples were placed in mixtures of Epon 812 and ethanol at concentrations of 25, 50 and 75% (60 min, room temperature). Then the samples were placed in 100% Epon 812 overnight at 277 K, and cured during a second night at 333 K.

Both $CaCl_2$ and $MgCl_2$ are known to decrease the loss of lipid during fixation of a variety of tissues (12). In the present work, $MgCl_2$ was chosen instead of $CaCl_2$ to avoid the formation of calcium phosphate precipitates. Osmium potassium ferrocyanide mixtures are increasingly popular as a secondary fixative in both conventional EM and in several cytochemical procedures because they combine selective fixation and enhance staining of membrane systems, glycocalix and glycogen (13–15).

Preparation of tissues by Procedure G + F. Samples were place in 0.8% formaldehyde (F) and 0.8% G in 0.075 M phosphate buffer, pH 7.2, for 1 h in darkness at 277 K. Samples were then washed for 2 h in PB at 277 K, which was followed by the dehydration and embedding procedures described in Procedure G + Os.

Preparation of tissues by Procedure G + TA + Os. Tissues were fixed in 1% G and 2 mg/ml tannic acid (TA) in PB for 30 min at room temperature in darkness. A brief washing in PB and 20-min post-fixation in 1% osmium tetroxide in PB at room temperature in the dark were followed by the same dehydration and embedding processes described in Procedure G + Os.

RESULTS

In our experiments, rats were injected i.p. with [^{3}H]CHO or [^{3}H]25-OH-CHO and the total radioactivity in the tissue and the fluids of the various steps of the different preparatory procedures was measured by liquid scintillation counting.

G, F and OsO_4 were used as fixatives and were applied according to standard procedures. However, our interest was mainly focused on the OsO_4 procedure, because OsO_4 or osmium, in combination with other agents such as TA or ferrocyanide, can reduce loss of some lipids in the course of dehydration and embedding steps.

Lipid metabolism. Lipid extracts of the liver and brain were analyzed by TLC one and five days after i.p. injection of [^{3}H]CHO. Most radioactivity was found in the TLC area corresponding to CHO (Figs. 1 and 2); between 6 and 10% was found in the area corresponding to sterol esters.

Nevertheless when the tracer injected was [^{3}H]25-OH-CHO, little radioactivity was found in TLC in the area corresponding to this compound (Figs. 1 and 2). One day after i.p. injection, radioactivity was found in all the scraped TLC areas and particularly in the region corresponding to the CHO standard (more than 20% in both aorta and brain). However, in the area corresponding to the labeled compound injected, less than 5% radioactivity was observed. When lipids were extracted five days after i.p. injection, practically all the radioactivity was at the origin in TLC. Evidently, the injected compound was converted into more polar products.

The percentage of tritium in the aqueous phase of the lipid extracts was also determined. In animals injected with [^{3}H]CHO, the percentage of tritium in liver and brain was 6 and 9%, respectively. When the tracer [^{3}H]25-OH-CHO was used, 53 and 43% of tritium was found in liver and brain, respectively, which suggests that [^{3}H]25-OH-CHO was metabolized to more polar compounds.

Radioactivity loss in the fixation procedures: [^{3}H]CHO. The total radioactivity loss in the procedures studied are summarized in Figures 3–6.

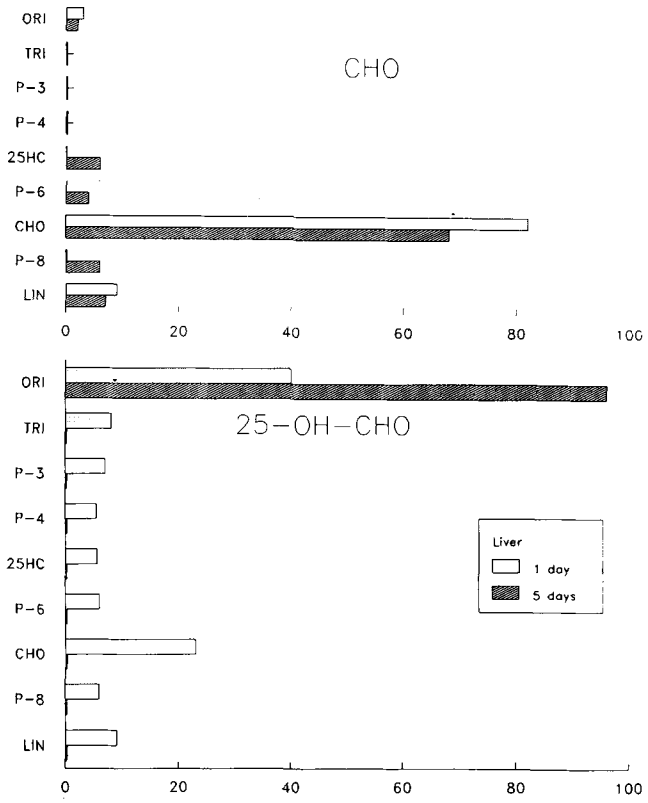

FIG. 1. Thin-layer chromatography profile of lipid extracts of liver one and five days after intraperitoneal injection with [1α,2α(n)-^{3}H]-cholesterol ([^{3}H]CHO) or 25-hydroxy[26,27-^{3}H]cholesterol ([^{3}H]25-OH-CHO). Values are percent of total radioactivity. The scraped areas corresponding to the four standards used are indicated as: TRI (cholestane-3β,5α,6β-triol), 25HC (cholestene-3β,25-diol), CHO (cholesterol) and LIN (cholesteryl linoleate); the areas corresponding to the origin and other positions are indicated as ORI and P-3 to P-8. Results are given as percentage of radioactivity found in each of the scraped areas, when the sum of all is taken to be 100%.

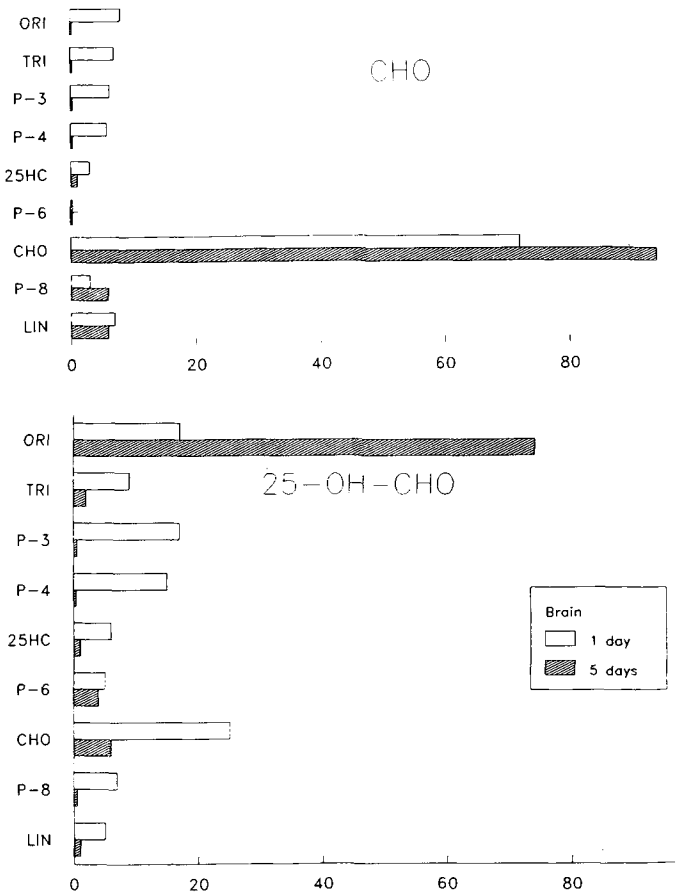

FIG. 2. Thin-layer chromatography of lipid extracts of brain one and five days after intraperitoneal injection with [^{3}H]CHO or [^{3}H]25-OH-CHO. Values are percent of total radioactivity. The scraped areas corresponding to the four standards used are indicated as: TRI (cholestane-3β,5α,6β-triol), 25HC (cholestene-3β,25-diol), CHO (cholesterol) and LIN (cholesteryl linoleate); the areas corresponding to the origin and other positions are indicated as ORI and P-3 to P-8. Results are given as percentage of radioactivity found in each of the scraped areas, when the sum of all is taken to be 100%. Abbreviations as in Figure 1.

To simplify the charts, the radioactivity loss measured in the pre-fixation, fixation, post-fixation and washing liquids are given in bulk as FIXAT; the sum of radioactivity losses in the dehydration fluids is given as DEHYD; and the losses for the different embedding steps are given together as EMBED.

None of the procedures studied prevented radioactivity loss in the liver (Figs. 3-6). The minimum loss observed was approximately 65% in the first two procedures, and essentially 100% in the third one. Radioactivity was lost mainly during the dehydration and embedding steps (k), while there was almost no loss of radioactivity during fixation.

By comparison, when aortic tissue was used, the percentage of radioactivity lost was 24 and 16% in the first two procedures, respectively, and 45% in the third one. Thus, there was a big difference between the results obtained for the liver (all procedures over 60% loss) and those obtained for the aorta (one of the procedures showed more than 80% retention of radioactivity).

Radioactivity loss in the fixation procedures: [^{3}H]25-OH-CHO. When [^{3}H]25-OH-CHO was used, the total loss of radioactivity in the three procedures was less than 20%; the results for the two types of tissue did not vary greatly. The loss of radioactivity in the fixation steps was much higher than with CHO, which is consistent with the conversion of the tracer into more polar compounds. The low loss of the polar radioactive metabolites during the dehydration and embedding steps is also consistent with the detected increase in polarity of the tritiated molecules.

DISCUSSION

During the last few years, special emphasis has been placed on EM preparatory procedures which retain functional properties of proteins throughout the fixation, dehydration and embedding steps (16). Conservation of protein functional features is particularly important in postembedding immunocytochemistry, which makes possible the analysis of the distribution, and in some cases the amount, of antigenic sites in tissues (17). Although the loss of lipids during preparatory procedures has been found not to affect the immunochemical technique, several functional properties of proteins depend on their lipid environment (18,19). In addition, loss of lipids, including

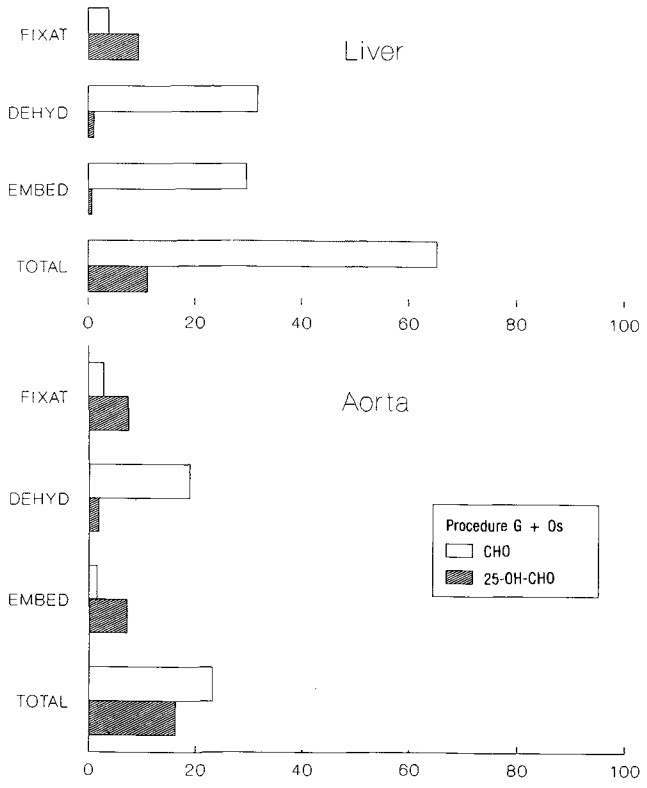

FIG. 3. Fixation procedure G + Os. Loss of radioactivity percentage during fixation of liver and aortic tissues five days after intraperitoneal injection with [^{3}H]cholesterol (CHO) or [^{3}H]25-OH-CHO. The radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXAT; the losses in the dehydration fluids are given as DEHYD; the losses in the embedding steps are given as EMBED, and the sum of the radioactivity losses are given as TOTAL. Abbreviations as in Figure 1.

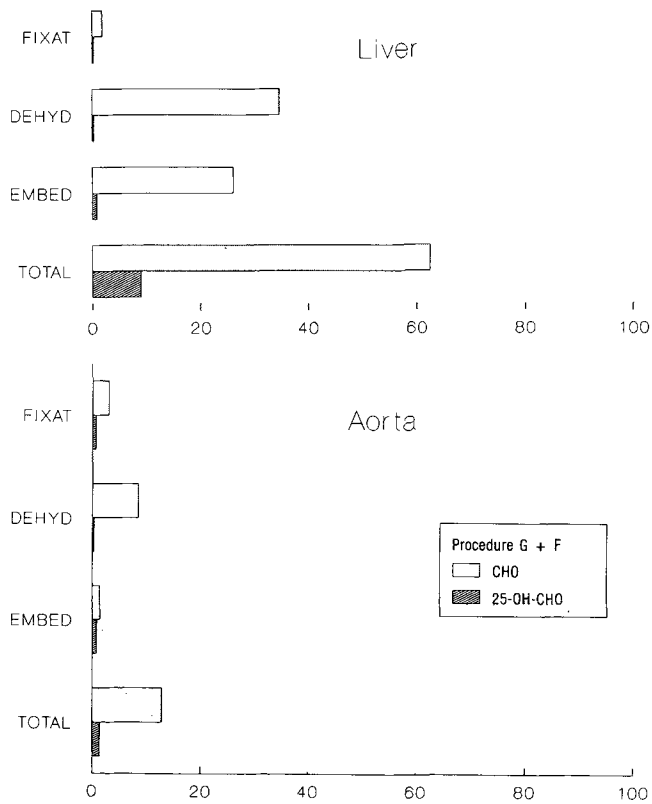

FIG. 4. Fixation procedure G + F. Loss of radioactivity percentage during fixation of liver and aortic tissues five days after intraperitoneal injection with[^{3}H]cholesterol (CHO) or [^{3}H]25-OH-CHO. The radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXAT; the losses in the dehydration fluids are given as DEHYD; the losses in the embedding steps are given as EMBED, and the sum of the radioactivity losses are given as TOTAL. Other abbreviations as in Figure 1.

CHO and CHO metabolites, during these preparatory procedures constitutes a disadvantage in studies on cell structures containing these molecules, such as plasma membranes. However, there are additional problems of CHO localization due to the ubiquitous presence of different chemical forms or the sterols in the cells. Free CHO forms part of the plasma membrane structure in mammalian cells, but CHO is typically stored as its ester and, finally is converted from its free form to other steroids. Our results indicating that both CHO and 25-OH-CHO are metabolized in tissues including liver and brain agree with those reported by Taylor and Kandutsch (20).

Most of the CHO loss from liver tissue occurred during the dehydration and embedding steps, whereas in aortic tissue the losses took place mainly during dehydration; very little radioactivity was lost in either tissue during the embedding steps. In contrast, the loss of 25-OH-CHO took place during fixation, which reflects the higher polarity of the tritium labeled metabolites of 25-OH-CHO as compared to those of CHO, as was demonstrated by TLC. The results suggest the need for alternative procedures to minimize the loss of both sterols.

Since dehydration at 277 K or partial dehydration was reported to reduce lipid loss (21,22), we analyzed in a preliminary study the effect of low temperature dehydration and embedding procedures on [^{3}H]CHO and [^{3}H]25-OH-CHO loss in liver and aortic tissues five days after i.p. injection of the tracers. The hydrophilic embedding medium Lowicryl K4M (Polysciences, Inc., Warrington, PA) was used for this purpose. The results show that this technique, used widely for immunocytochemistry, also significantly reduces the loss of both sterols. The total loss of CHO in liver and aortic tissues was 23 and 7%, respectively, while 6 and 4% of 25-OH-CHO metabolites were lost in liver and aortic tissues, respectively. Therefore, low temperature procedures recommend themselves as superior alternatives to standard EM procedures. However, other more recent techniques, such as cryofixation, also need to be investigated.

Our results further show that the loss of CHO differs from that of CHO derivatives, such as 25-OH-CHO. For this reason, the results obtained for CHO fixation cannot be extrapolated for other steroids. Moreover, there is no standard procedure for preventing the loss of CHO from different mammalian tissues during EM preparation. The loss of CHO appears to depend on several factors, including its chemical form in which it is present in these tissues.

The role of OsO_4 in the retention of lipids during fixation remains unclear. Whereas in some studies it has been

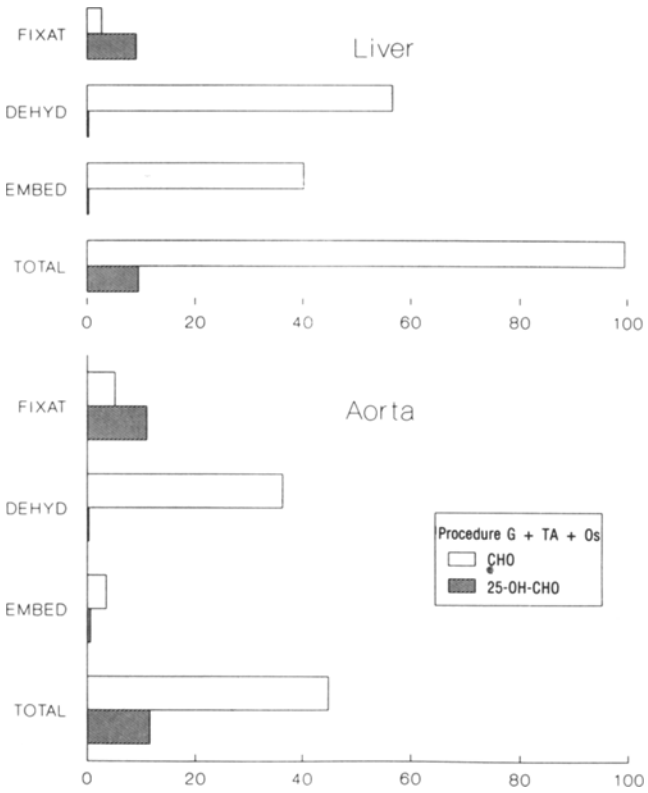

FIG. 5. Fixation procedure G + TA + Os. Loss of radioactivity percentage during fixation of liver and aortic tissues five days after intraperitoneal injection with [^{3}H]cholesterol (CHO) or [^{3}H]25-OH-CHO. The radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXAT; the losses in the dehydration fluids are given as DEHYD; the losses in the embedding steps are given as EMBED, and the sum of the radioactivity losses are given as TOTAL Other abbreviations as in Figure 1.

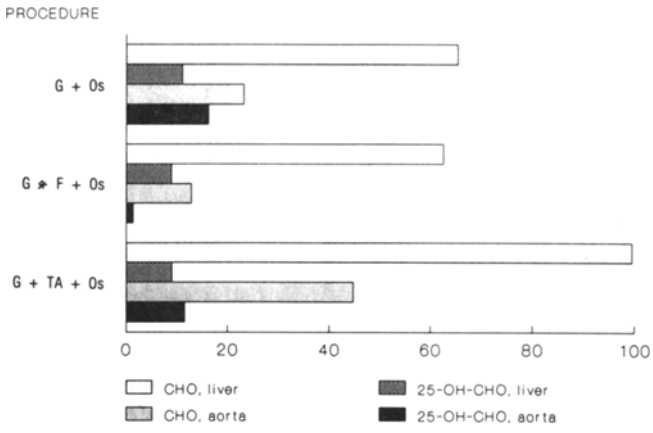

FIG. 6. Total losses of radioactivity percentage during fixation of liver and aortic tissues by Procedures G + Os, G + F and G + TA + Os, five days after intraperitoneal injections with [^{3}H]cholesterol (CHO) or [^{3}H]25-OH-CHO. Other abbreviations as in Figure 1.

shown that this fixative minimizes lipid loss (2), x-ray diffraction analyses indicate that fixation with this compound is not enough to prevent the loss of CHO during dehydration (23).

The staining system osmium ferrocyanide is widely used to enhance staining of membranes and the sarcoplasmic reticulum. There is an equilibrium between the different oxidation states, Os(VII) and Os(VI), where Os is immobilized at the chelation sites of the macromolecular matrix of the substrate (13). Little is known about this process, but it seems that while the bilayer lipids disappear, remnants of the former structure (probably denatured proteins) remain, although much of the protein is also lost (24).

Inclusion of osmium in the fixation process does not affect the structures visualized, and the effect on proteins is the same whether there has been prefixation or not. Making proteins insoluble with TA also does not significantly prevent protein loss (24). In fact, there seems to be no relationship between the proposed mechanisms for osmium staining and the loss of CHO in the fixation procedures studied. Our data on CHO retention in liver tissue show that the use of $OsO_4/K_4Fe(CN)_6$ (Procedure G + Os) gave the same result as Procedure G + F (0.8% F + 0.8% G, without osmium treatment). The best results with aortic tissue were obtained with Procedure G + F. The loss of CHO was the highest in both liver and aortic tissues with Procedure G + TA + Os (TA in the fixative solution and OsO_4 treatment), even though TA is a chelating agent for osmium (13).

When 25-OH-CHO was used as tracer, the loss of tritium was lower than when CHO was used, and the use of osmium did not increase metabolite retention.

In summary, our results show that the degree of CHO and 25-OH-CHO loss does not depend only on the procedures used for EM but also on the type of tissue. This is in substantial agreement with previous studies that have focused on total lipids (2,21).

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Cholesterol and 25-Hydroxycholesterol Retention in Specimens of Liver and Aorta Prepared for Electron Microscopy. II. Effect of Filipin, Osmium, Digitonin and Saponin

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Sprague-Dawley rats were injected intraperitoneally with [1α,2α(n)-^{3}H]cholesterol or 25-hydroxy-[26,27-^{3}H]cholesterol, and one and five days later liver and aortic tissues were fixed. The extent to which these sterols were lost from the tissues during preparation for electron microscopy (EM) was examined utilizing different fixation procedures and various protective agents. Radioactive tracers, scintillation counting and standard EM techniques were used. Although most of the procedures examined caused major lipid losses, useful fixation procedures that allow retention of cholesterol or 25-hydroxycholesterol in liver and aortic tissues were found and are described here.
Lipids 28, 929–935 (1993).

Lipids usually constitute one of the major components of cell membranes, and in most mammalian membranes cholesterol (CHO) is present at an about equimolar ratio with phospholipids. One of the limiting factors in plasma membrane studies using electron microscopy (EM) is that the conventional procedures for preparing biological material for EM lead to extensive loss of lipids (1). In the preceding paper we examined the loss of CHO and of 25-hydroxy-cholesterol (25-OH-CHO) from rat aorta and liver tissues during conventional EM preparatory procedures (2). The procedures included aldehyde fixation with or without osmium tetroxide postfixation, dehydration in ethanol, and embedding in Epon 812. We found that the radioactive tracer 25-hydroxy-[26,27-^{3}H]cholesterol ([^{3}H]25-OH-CHO), when injected intraperitoneally (i.p.), was metabolized to more polar compounds. When the tracer injected was [1α,2α(n)-^{3}H]cholesterol ([^{3}H]CHO), about 90% of the radioactivity of the lipid extracts was associated with the CHO fraction, and about 10% was converted to steryl esters. The calculated increases in CHO present in liver and aortic tissues upon injection of the tracer were 5.3×10^{-4} and 3.6×10^{-4}%, respectively. Under these conditions loss of CHO significantly differs from that of its derivatives. Our results showed, moreover, that CHO loss from mammalian tissues cannot be avoided when conventional EM procedures are used. This appears to be due to several factors, including the different chemical forms into which CHO is converted in a particular tissue. Our results also pointed out the importance of finding alternative procedures which would make it possible to analyze by EM cell structures containing CHO.

The aim of the present work was to compare various procedures used in CHO cytochemistry and to examine how CHO and 25-OH-CHO loss and total lipid loss can be prevented during the preparation of the biological specimens for EM. The procedures we examined include addition of digitonin, saponin or filipin to the fixative solutions in an attempt to preserve CHO in its native location. The experiments were carried out by giving rats i.p. injections of [^{3}H]CHO and [^{3}H]25-OH-CHO and by measuring the radioactivity present in the residual fluids of the various steps of the preparatory process.

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Abbreviations: CB, cacodylate buffer; CHO, cholesterol; [^{3}H]CHO, [1α,2α(n)-^{3}H]cholesterol; EM, electron microscopy; F, formaldehyde; G, glutaraldehyde; i.p., intraperitoneal(ly); 25-OH-CHO, 25-hydroxycholesterol; [^{3}H]25-OH-CHO, 25-hydroxy-[26,27-^{3}H]cholesterol; PB, 0.1 M phosphate buffer (pH 7.3, 2 mM $MgCl_2$).

MATERIALS AND METHODS

Animals and tracers. Sprague-Dawley rats weighing 150–200 g were used. One and five days prior to being sacrificed, animals were injected intraperitoneally with 5 mCi of [^{3}H]CHO, 40–60 Ci/mmol (Amersham International, Amersham, England), or 2 mCi of [^{3}H]25-OH-CHO, 75–87 Ci/mmol (New England Nuclear, Boston, MA). Both products were prepared for injection as described by Baranowsky *et al.* (3). After evaporation of the toluene from the original vial containing the tracer, the labeled sterol was redissolved in 0.5 mL of ethanol, 1 mL of saline was added to this solution and most of the ethanol was evaporated in a stream of nitrogen. The resulting sterol suspension was injected i.p.

For removal of the tissues, the rats were kept under pentobarbital anesthesia, and after cannulating the aorta, saline was infused at 100 mm Hg for one minute. Immediately thereafter, aliquots of tissue from aorta and liver were taken for each of the fixation procedures, which were started immediately. Aliquots of aorta and liver were also taken to measure radioactivity per tissue.

Counting techniques. Radioactivity of the residual liquids of the individual steps of the fixative procedures was measured. As the chemical composition of the samples varied greatly, the counting efficiency for each type of sample was determined based on internal standards, and the counts were converted into disintegrations per min (dpm). Special care was required to measure the samples containing osmium, which had especially low counting efficiencies. The original dpm of the tissues to be fixed was determined by dissolving aliquots of the same type of tissue in Nuclear Chicago Solubilizer (Chicago, IL) and counting as described before. A toluene/Triton X-100 liquid scintillator (Scharlau, Barcelona, Spain) and LKB 1219 Rackbeta counter (LKB, Turku, Finland) were used. Results are the mean of three experiments; three animals were injected with each tracer, and two samples of tissue per animal were fixed independently for each of the fixative procedures studied.

Radioactivity was measured in each of the fluids used in all the processes, but in order to simplify the charts, the radioactivity losses measured in the pre-fixation, fixation, post-fixation and washing liquids are given in bulk as FIXATION, the sum of radioactivity losses in the

dehydration fluids is given as DEHYDRATION, and the losses in the different embedding steps are given together as EMBEDDING.

Radioactivity calculations. Since the weight of the aliquots of tissue to be fixed (and consequently their total radioactivity) was not uniform, they were weighed and their radioactivities calculated using the values given in Table 1. The radioactivity losses are given in percentages to facilitate comparison of the different fixation processes.

The radioactivity remaining in the fixed aliquots of tissue is taken to be the difference between the total radioactivity of the aliquot (calculated) and the sum of radioactivity losses measured in the fluids. The embedded aliquots were then used to obtain representative electron micrographs.

Preparation of tissues for EM. General procedure. Aliquots of tissue from aorta (3–4 mg) and liver (1–4 mg) were immersed for different periods of time (4) into 0.1 M phosphate buffer (PB) (pH 7.3, 2 mM $MgCl_2$) or in cacodylate buffer (CB) (pH 7.3, 2 mM $MgCl_2$) containing 1% glutaraldehyde (G) following the specific procedures described below. Effective osmolarity of the fixative solution (300 mOsm) was determined as described in the literature (5,6). The purity of G (Merck, Darmstadt, Germany) was determined by measuring the relative ultraviolet absorbances between 210 and 310 nm (5,7,8); the spectra showed a peak only at 280 nm. After brief washing in PB, the tissues were postfixed with 2% OsO_4 containing 1.8% potassium ferrocyanide in PB for 2 h in the dark at room temperature (296 K).

Samples were dehydrated by 5-min immersions into ethanol (60, 80 and 95%)/water (vol/vol), and by two 5-min immersions into 100% ethanol; all immersions were at 273 K. Use of propylene oxide was not considered necessary.

Epon 812 (Polysciences, Warrington, PA) consisted of 21 mL Poly/Bed 812, 13 mL dodecenyl succinic anhydride, 11 mL nadic methyl anhydride and 0.7 mL DMP-30, which was added just before use. Samples were placed into mixtures of Epon 812 and ethanol at concentrations of 25, 50 and 75% (60 min, room temperature). Then the samples were placed in 100% Epon 812 overnight at 277 K, and cured during a second night at 333 K.

Both $CaCl_2$ and $MgCl_2$ are known to decrease the loss of lipid during fixaton of various tissues (9). In the present work, $MgCl_2$ was used instead of $CaCl_2$ to avoid formation of calcium phosphate precipitates. Osmium/potassium ferrocyanide mixtures are increasingly popular as a secondary fixative in both conventional EM and in various cytochemical procedures because they combine selective fixation and enhanced staining of membrane systems, glycocalix and glycogen (10–12).

Preparation of tissues for EM. Specific procedure. CHO was localized in membranes by using saponin, digitonin or polyene antibiotics such as filipin (13,14). The latter reagent forms large complexes with CHO that are visible in freeze-fracture replicas as distinct ondulations in thin sections or protuberances. Use of filipin is the method of choice for cytochemical localization of sterols (14) and is also used in studies that require blocking of membrane steroids (15,16).

We therefore examined the ability of these substances to retain CHO or 25-OH-CHO in the course of the EM preparatory procedures in the tissues. The G fixation step is the only one studied here that is relevant to freeze-fracture preparations (14). The other steps are required in the preparation for thin-sectioning.

Filipin treatment (Procedures 1 and 2). Unfractionated filipin (Upjohn Co., Kalamazoo, MI) was added to the G-based fixatives in CB (see general procedure section) at concentrations of 0, 2, 5, 10, 20, 60 and 100 μg/mL. Filipin was first dissolved in dimethyl sulfoxide and then added to the fixative (final concentration of dimethyl sulfoxide was 1% in all solutions). Fixation was for 24 h at room temperature in the dark. Samples were then processed for EM as described in the general procedure. In the pre-treatment with filipin (Procedure 2), tissues were incubated before fixation for 2 h at room temperature and in the dark in CB containing filipin at concentrations of 100, 10 and 0 μg/mL. Thereafter, the general procedure was followed.

The ability of filipin to form complexes with β-hydroxysterols had been tested in a previous study (17) using freeze-fracture on synaptosomes isolated from cerebral cortex of rats.

Osmium tetroxide treatment (Procedure 3). Since mixtures of aldehydes and Os have been recommended for different types of tissues, isolated cells and cells in monolayers (2,18,19), samples were fixed in 1% osmium tetroxide, 0.8% formaldehyde (F) and 0.8% G in 0.075 M PB, pH 7.2 for 1 h in the dark at 277 K. A similar fixative but without osmium tetroxide was used in parallel, and the

TABLE 1

Radioactivity in Liver and Aortic Tissues Five Days After i.p. Injection with [^{3}H]CHO or [^{3}H]25-OH-CHO[a]

	dpm/mg tissues		mmol [^{3}H]CHO/mg		% CHO increase	
Animal number	Liver	Aorta	Liver	Aorta	Liver	Aorta
Tracer: [^{3}H]CHO						
1	6 030	7 740				
2	2 551	1 686				
3	9 340	1 300				
Means	5 973	3 575	5.4×10^{-11}	3.2×10^{-4}	5.3×10^{-4}	3.6×10^{-3}
Tracer: [^{3}H]25-OH-CHO						
4	1 973	9 550				
5	1 082	813				
6	1 128	890				

[a]Values of [1α,2α(n)-^{3}H]cholesterol ([^{3}H]CHO) in mmol/mg of tissue and the cholesterol (CHO) increases were estimated as described in Counting Techniques. 25-Hydroxy-[26,27-^{3}H]cholesterol ([^{3}H]25-OH-CHO) values were not calculated, as this tracer is rapidly metabolized. i.p., intraperitoneal.

two procedures are identified as 3-Osmium and 3-Osmium Blank. Samples were then washed for 2 h in PB at 277 K, and dehydrated and embedded as described in the general procedure.

Digitonin treatment (Procedure 4). A partial dehydration method, similar to the one described by Darrah *et al.* (20), was followed. Samples were fixed for 1 h in 2.5% G and 0.2% digitonin in PB at 277 K, and in the corresponding fixative without digitonin. The two procedures are identified as 4-Digitonin and 4-Digitonin Blank. After one-hour post-fixation in Dalton's chrome/osmium tetroxide, the tissues were dehydrated at 273 K, 5 min each step, in two 70% ethanol steps, and in two 95% ethanol steps. This was followed by four embedding steps with 100% Epon 812 at 277 K. The first three were for one hour each and the last one continued overnight. Finally the sample was embedded in Epon 812 for 90 min at 310 K and cured at 333 K overnight.

Saponin treatment (Procedure 5). Tissues were fixed in 1% G, 2 mg/mL tannic acid and 0.5 mg/mL saponin in PB for 30 min in the dark at 293 K. A corresponding control without saponin was also used. The two procedures are identified as 5-Saponin and 5-Saponin Blank. A brief washing in PB and 20-min post-fixation in 1% osmium tetroxide in PB at room temperature in the dark were followed by dehydration and embedding as described in the general procedure.

RESULTS

Table 1 gives the radioactivities found in liver and aortic tissues, the values in mmol of the tracer [^{3}H]CHO and the estimated CHO increase due to the tracer. CHO increases (5.3×10^{-4}% for liver and 3.6×10^{-3}% for aorta) were calculated using 3.9 g/kg and 1.0 g/kg for the CHO contents of liver and aortic tissues, respectively (21). Increases in [^{3}H]25-OH-CHO were not evaluated, as this tracer is rapidly metabolized and the radioactivity found in the tissues corresponds to other, more polar compounds (2).

Treatment of tissue with osmium tetroxide (Procedure 3 and the corresponding blank procedure) resulted in protection against loss of CHO (13 and 14%, respectively) and 25-OH-CHO and its metabolites (1 and 1.4%, respectively) from aortic tissue (Figs. 1 and 2). However, in the liver only 25-OH-CHO and its metabolites (9.2 and 9.0% radioactivity loss, respectively) were preserved when this treatment was used (Figs. 3 and 4).

When the specific cytochemical procedures were used, *i.e.*, with inclusion of filipin, digitonin or saponin, the results varied greatly. None of the filipin procedures prevented CHO loss from the liver, and in all cases losses were over 60% (Fig. 5). In all the filipin procedures, in fact, the results were quite similar to those of the controls, which shows that filipin provides little protection. When filipin was used in the first step (but without G) to fix CHO or 25-OH-CHO in the liver, the radioactivity loss was as high as in the standard filipin procedures (over 60% loss for CHO and between 7 and 12% for 25-OH-CHO and its metabolites). The results of the control procedure were also quite similar to the results of the filipin procedures (Figs. 6 and 8). The results, however, were clearly different when CHO was fixed with filipin in the aortic tissue, where the total radioactivity loss was reduced (Fig. 7), but still was more than 15%.

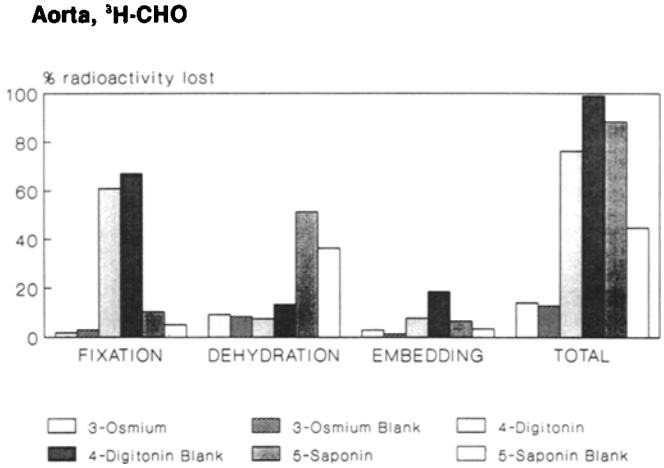

FIG. 1. Fixation Procedures 3, 4 and 5 (treatments with osmium tetroxide, digitonin and saponin). Loss of radioactivity during fixation of aortic tissue five days after intraperitoneal injection of [^{3}H]cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

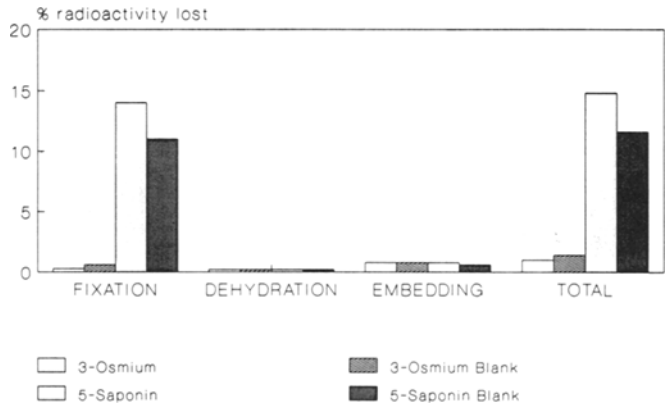

FIG. 2. Fixation Procedures 3 and 5 (treatments with osmium tetroxide and saponin). Loss of radioactivity during fixation of aortic tissue five days after intraperitoneal injection of [^{3}H]25-OH-cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

All the filipin procedures tested on liver tissue resulted in a smaller radioactivity loss in the case of 25-OH-CHO (between 7 and 12%) than for CHO (over 60%; Figs. 5, 6 and 8), and similar results were obtained in aortic tissue (losses close to 20% for CHO and close to 15% for 25-OH-CHO and its metabolites; Figs. 7 and 9). The total loss, however, was high in all the procedures, and again control and filipin procedures gave similar results. Results obtained in pre-treatment with filipin were good in the case of 25-OH-CHO and its metabolites showing close to 7% retention in liver (Fig. 6), and, again, the blank procedure gave results similar to those obtained with filipin.

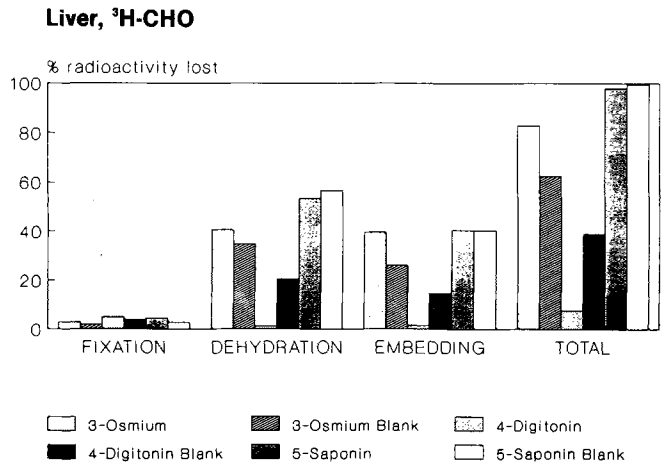

FIG. 3. Fixation Procedures 3, 4 and 5 (treatments with osmium tetroxide, digitonin and saponin). Loss of radioactivity during fixation of liver tissue five days after intraperitoneal injection of [^{3}H]cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

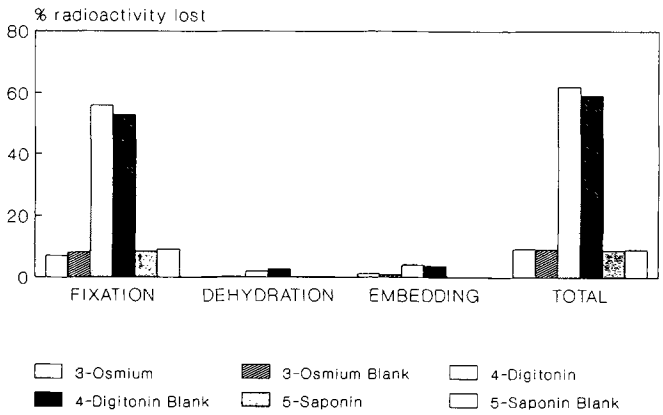

FIG. 4. Fixation Procedures 3, 4 and 5 (treatments with osmium tetroxide, digitonin and saponin). Loss of radioactivity during fixation of liver tissue five days after intraperitoneal injection of [^{3}H]25-OH-cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

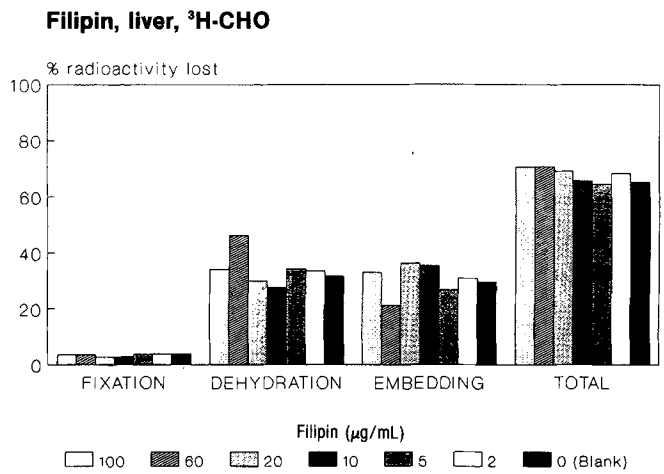

FIG. 5. Fixation Procedure 1 (treatment with filipin). Radioactivity loss in liver tissue fixation five days after intraperitoneal injection of [^{3}H]cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

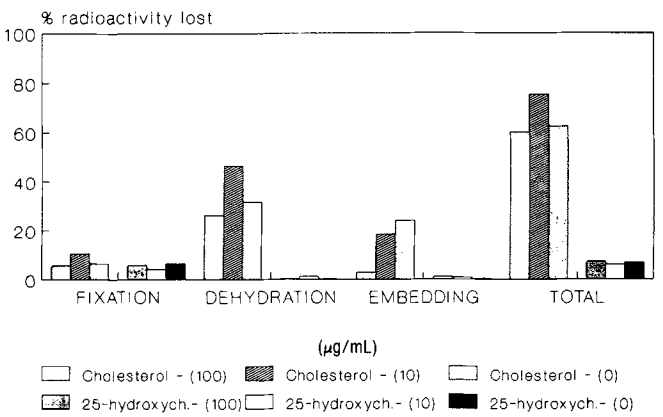

FIG. 6. Fixation Procedure 2 (pre-treatment with filipin). Radioactivity losses in liver tissue fixation five days after intraperitoneal injection of [^{3}H]cholesterol (CHO) and [^{3}H]25-OH-CHO. Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL. Abbreviation: 25-hydroxych., 25-hydroxycholesterol.

Digitonin, which has been described as a good tool for avoiding lipid losses, protected liver against CHO loss (7.5% loss *vs.* 39% with the corresponding blank procedure, Fig. 3). However, in aorta tissue more than 75% of CHO was lost using the digitonin procedure and its blank (Fig. 1). Digitonin does not prevent 25-OH-CHO loss from liver (losses of more than 60% for the procedure and its blank, Fig. 4).

In contrast, saponin treatment and the corresponding blank procedure prevented losses of 25-OH-CHO and its metabolites in both aorta and liver tissues (12 and 9% loss, respectively, Figs. 2 and 4).

DISCUSSION

In recent years many attempts have been made to develop EM preparatory procedures which minimize the loss of molecules from the specimens and preserve structural integrity and functional properties (2,9,20,22). Special emphasis has been placed on procedures which retain the antigenicity and/or enzymatic activity of a variety of proteins (20,22,23). Although it has been claimed that the loss of lipids is not a serious problem (22), it constitutes an important disadvantage in studies on the ultrastructural

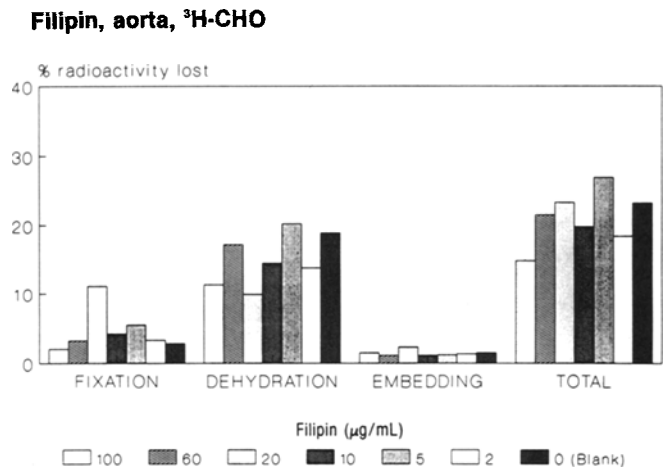

FIG. 7. Fixation Procedure 1 (treatment with filipin). Loss of radioactivity during fixation of aortic tissue five days after intraperitoneal injection of [^{3}H]cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

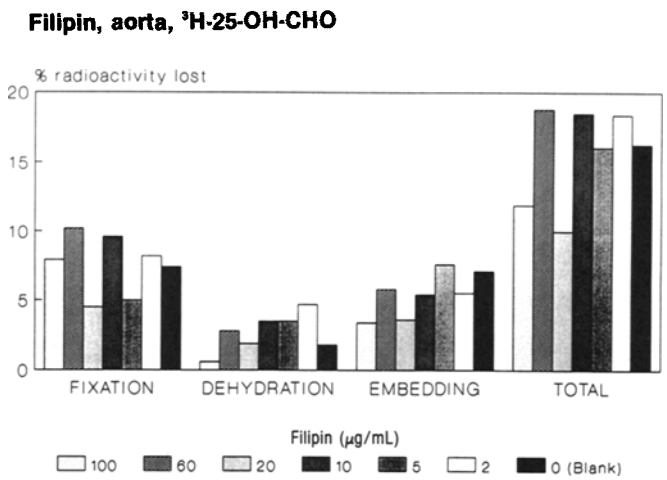

FIG. 9. Fixation Procedure 1 (treatment with filipin). Loss of radioactivity during fixation of aortic tissue five days after intraperitoneal injection of [^{3}H]25-OH-cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

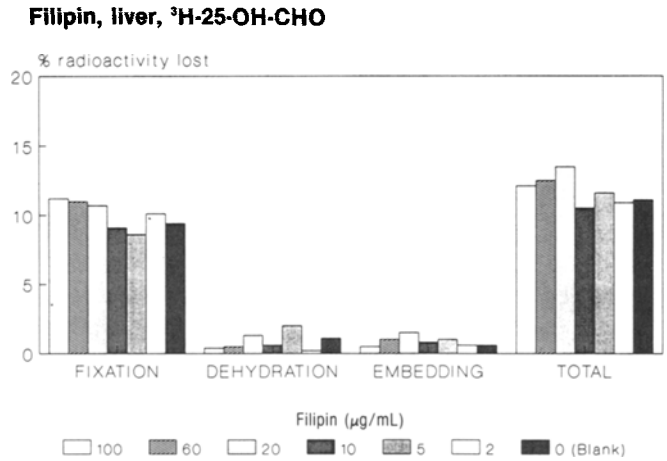

FIG. 8. Fixation Procedure 1 (treatment with filipin). Loss of radioactivity during fixation of liver tissue five days after intraperitoneal injection of [^{3}H]25-OH-cholesterol (CHO). Radioactivity losses during pre-fixation, fixation, post-fixation and washing are given in bulk as FIXATION; losses in all the dehydration fluids are given as DEHYDRATION; losses in all the embedding steps are given as EMBEDDING; and the sum of all the radioactivity losses are given as TOTAL.

localization of CHO. Moreover, there are important additional problems of CHO localization due to the ubiquitous presence of different sterol forms in the cells. Free CHO forms part of the plasma membrane structure in mammalian cells, CHO is stored as steryl ester, and, finally, it is metabolized to other steroids.

The losses of [^{3}H]CHO introduced in the tissues (estimated CHO increases of 5.3×10^{-4}% for liver and 3.6×10^{-3}% for aorta) are used to evaluate CHO retention. We assumed that the tracer is uniformly distributed in the tissues and that its loss corresponds to the loss of native CHO, although this has not been proven. It also must be taken into account that the radioactivity losses from tissues of animals injected with [^{3}H]25-OH-CHO largely correspond to 25-OH-CHO metabolites and not to native 25-OH-CHO, if present at all.

The staining system osmium/ferrocyanide is widely used for enhanced staining of membranes and of the sarcoplasmic reticulum. There is an equilibrium between the different oxidation states, Os(VII) and Os(VI), that are immobilized at the chelation sites of the macromolecular matrix of the substrate (9). Little is known about this process, but it seems that while the lipid bilayers disappear, remnants of the former structure (probably denatured proteins) remain, although much of the protein is lost (24). Our results confirm that osmium does not retain CHO in the tissues studied, where the same results were obtained for osmium containing fixative and the corresponding blank (Figs. 1–4).

When digitonin was used as a protective agent (19) our results for CHO retention in liver coincided with those reported by Scallen and Dietert (25) for this tissue and by Darrah *et al.* (20) for the lung. Moreover, thin sections obtained from liver tissue fixed in the presence of digitonin revealed good ultrastructural preservation of hepatocyte components (Fig. 10A). By contrast, digitonin treatment does not prevent 25-OH-CHO metabolite loss from the liver. Also, a significant loss of CHO was observed in aortic tissue when digitonin was used during aldehyde fixation. This suggests that digitonin does not stabilize CHO in this tissue, although the ratio of free CHO *vs.* esterified CHO is similar to that observed in the liver. The results obtained with [^{3}H]25-OH-CHO in the liver very much depend on the type of fixative and buffer used.

Pretreatment of tissue with filipin before fixation and the corresponding blank procedure retain about 90% of the 25-OH-CHO metabolites. However, filipin treatment of unfixed tissue causes extensive alterations in cell ultrastructure, including mitochondrial disorganization

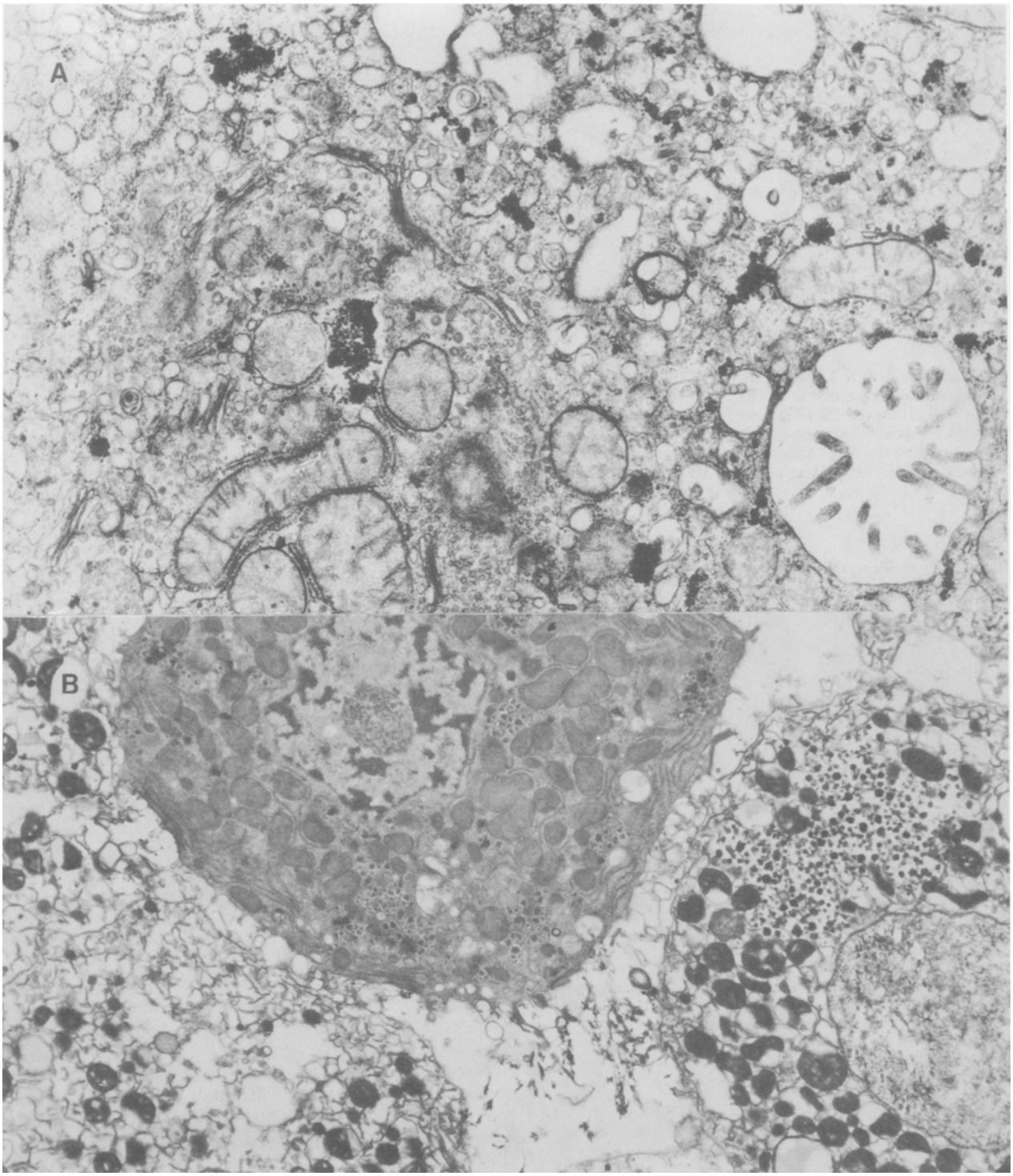

FIG. 10. (A) Details of a rat hepatocyte processed for electron microscopy using digitonin as a protective agent (Procedure 4). There is a good preservation of the ultrastructure of most of the cell components, including mitochondria, endoplasmic reticulum, glycogen and small vesicles. Some specific alterations induced by this treatment are a collapsed Golgi apparatus and the presence of some clear vacuoles. (B) Micrograph showing the effect of pretreatment of rat liver with filipin before fixation. As shown, this treatment induces an extensive alteration of hepatocyte ultrastructure, including mitochondrial disorganization. (A, ×8585.60; B, ×28981.60.)

and swelling (Fig. 10B). This finding is consistent with reported results (12).

Some of the procedures tested here could be used in conventional EM, and in cytochemical or autoradiographic studies on the ultrastructural localization of CHO or CHO derivatives. Thus, the combination of G and F significantly reduces CHO and 25-OH-CHO loss in liver and in aortic tissue. Moreover, this procedure results in a good ultrastructural preservation of cell components (18) and can be used in ultrastructural studies concerned with CHO distribution by freeze-fracture techniques using filipin as marker.

Although the procedures described here and in the preceding paper (2) are widely used in conventional EM as well as in cytochemistry, various procedures have been developed during the last few years that would avoid problems such as loss of antigenicity and functional activities and also just loss of mass. These procedures include cryofixation and the use of low temperature embedding resins. Although the cryofixation techniques appear to be a good alternative to standard techniques, technical problems still prevent their routine use. In contrast, low temperature embedding appears to become an established method facilitating cytochemical and immunochemical procedures. Moreover, these latter methods result in good ultrastructural resolution as well as provide suitable contrast. Preliminary results obtained in our laboratory have shown that low temperature embedding resins, such as Lowicril K4M, are a good tool and minimize sterol loss in the preparatory procedures for thin sectioning.

ACKNOWLEDGMENTS

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The Effect of Lyophilization on the Solvent Extraction of Lipid Classes, Fatty Acids and Sterols from the Oyster *Crassostrea gigas*

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The lipid class compositions of adult Pacific oysters [*Crassostrea gigas* (Thunberg)] were examined using latroscan thin-layer chromatography/flame-ionization detection (TLC/FID), and fatty acid compositions determined by capillary gas chromatography and gas chromatography/mass spectrometry (GC/MS). The fatty acid methyl esters were separated using argentation TLC and also analyzed as their 4,4-dimethyloxazoline derivatives using GC/MS. Major esterified fatty acids in *C. gigas* were 16:0, 20:5n-3, and 22:6n-3. C_{20} and C_{22} nonmethylene interrupted (NMI) fatty acids comprised 4.5 to 5.9% of the total fatty acids. The NMI trienoic fatty acid 22:3(*7,13,16*) was also identified. Very little difference was found in the proportions of the various lipid classes, fatty acids or sterols between samples of adult oysters of two different sizes. However, significant differences in some of the lipid components were evident according to the method of sample preparation used prior to lipid extraction with solvents. Lyophilization (freeze drying) of samples led to a significant reduction in the amounts of triacylglycerols (TG) extracted by solvents in two separate experiments (7.0 and 52.5% extracted). Extracts from lyophilized samples had less 16:0, C_{18} unsaturated fatty acids, and 24-ethylcholest-5-en-3β-ol, while C_{20} and C_{22} unsaturated fatty acids comprised a higher proportion of the total fatty acids. There was no significant change in the amounts of polar lipids, total sterols, free fatty acids or hydrocarbons observed in extracts from lyophilized samples relative to extracts from nonlyophilized samples. Addition of water to the freeze-dried samples prior to lipid extraction greatly improved lipid yields and resulted in most of the TG being extracted.
***Lipids* 28, 937-944 (1993).**

Species of true oyster (family Ostreidae), primarily of the genera *Crassostrea* and *Ostrea* are intensively grown in mariculture farms for human consumption worldwide. The economic value of these bivalve molluscs has resulted in considerable efforts to improve production by increasing survival and growth rates, especially of the larval stages. Insufficient quantities of the long-chain polyunsaturated fatty acids (PUFA) 20:5n-3 and 22:6n-3 in the diet can lead to reduced growth rates and increased mortality in larval and juvenile bivalve molluscs (*e.g.*, 1-3). Larval viability has also been related to the total lipid content of oyster eggs and larvae (4,5), and variations in total lipid with age and season in adult oysters can give a measure of the mollusc's nutritional and reproductive status (6,7). As in most marine animals, the polar lipids (PL) in species of *Crassostrea* contain relatively more PUFA than neutral lipids [primarily triacylglycerols (TG)] (2,8,9). Because these lipid classes are not homogeneous with respect to fatty acid composition, incomplete lipid extraction would result in incorrect estimates of total lipid content and PUFA composition and also of larval condition and adult reproductive status where such lipid indices are used (5).

One of the most widely used techniques for solvent extraction of lipid from animal tissues is the method of Bligh and Dyer (10). This rapid method was developed for the extraction of lipids from fresh and frozen fish flesh, yielding approximately 94% of the total lipid or even more if the residue is reextracted. The authors suggested it could be used for many other tissues, and over the years this technique has been adapted successfully for the extraction of lipids from a wide range of terrestrial and aquatic samples. Variable results for lipid extraction using alternative solvent systems have been reported and appear to be highly tissue dependent (*e.g.*, 11-13).

The lyophilization or freeze drying of samples is a common technique employed as a form of preservation of animal tissues prior to analysis, and is useful in sample preparation as it makes tissues brittle and easily homogenizable and can be beneficial by denaturing enzymes (14). It has long been recognized that lyophilization increases the difficulty with which lipids can be extracted with solvents (*e.g.*, 14,15), and even though it is a widely used technique, the effect of this form of preservation on the efficiency of solvent extraction of lipids from tissues is rarely established. Lyophilization can also lead to the loss of certain compounds (16) and to artifact formation (17). Many schemes have been proposed involving lyophilization of samples prior to analysis of lipids as well as cellular protein, carbohydrate and nucleic acids (18-20), where lyophilization presumably does not affect the extraction of these components.

This study examined the effect of lyophilization on the solvent extraction of lipids from adult Pacific oysters (*Crassostrea gigas*) using a modification of the method of Bligh and Dyer (10). Also described is a modification of the method of Fay and Richli (21) for 4,4-dimethyloxazoline (DMOX) derivatization, which permits the rapid identification of double-bond positions in PUFA without loss of unsaturated fatty acids.

MATERIALS AND METHODS

Samples and lipid extraction. Lipids were extracted using a modification of the method of Bligh and Dyer (10). Solvent ratios from the original method were retained, *i.e.*, monophasic extraction with chloroform/methanol/water (1:2:0.8, by vol), followed by partition of aqueous and organic phases by solvent ratio adjustment to

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Abbreviations: DG, diacylglycerols; DMDS, dimethyldisulfide; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; FFA, free fatty acids; FID, flame-ionization detector; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; NMI, nonmethylene interrupted; NMID, nonmethylene interrupted dienoic fatty acid; NMIT, nonmethylene interrupted trienoic fatty acid; PL, polar lipids; PUFA, polyunsaturated (polyenoic) fatty acids; TG, triacylglycerols; TLC, thin-layer chromatography; TLC/FID, thin-layer chromatography/flame-ionization detection; TMTD, trimethyltridecanoic acid; WE, wax esters.

chloroform/methanol/water (1:1:0.9, by vol). Significant changes to the original method were the increased solvent–residue contact time (from 10 min to overnight; Experiment 1), or increasing the number of solvent washes of the residue (from 2 to 4) and incorporating ultrasonication to aid in cell membrane disruption at each solvent–residue stage (Experiment 2). The Bligh and Dyer method (10) with either modification yielded the same total lipid and lipid class composition for duplicate samples of wet tissue of adult *C. gigas* (Dunstan, G.A., and Volkman, J.K., unpublished data). All organic solvents used were Nanograde® (Mallinckrodt, Paris, KY). All water used in these and subsequent steps was purified using a Milli-Q® Type I Reagent Water System (Millipore®, Bedford, MA) which removes dissolved inorganic electrolytes, particles (<0.22 μm) and organic compounds. Blank extractions were also performed to establish the lack of contaminants.

Experiment 1. Oysters of similar genetic stock and exposed to similar environmental conditions were collected out of breeding season (winter) from Pipe Clay Lagoon, Tasmania, Australia. Twelve adult oysters each of two size classes, large (average soft tissue weight 8.9 ± 0.5 g) and small (average soft tissue weight 5.8 ± 0.4 g), were divided into two replicate batches of six oysters each and frozen in liquid nitrogen prior to analysis. One batch of the large oysters and one batch of the small oysters were chopped and then homogenized in solvent separately with a Ystral® (Döttingen, Germany) homogenizer. These "control" samples (solvent extracted nonlyophilized wet tissue) were left to extract overnight in chloroform/methanol/water (1:2:0.8, by vol; 250 mL), then partitioned against chloroform/water (1:1, vol/vol) (taking tissue water content into account) to give a final solvent ratio of chloroform/methanol/water of 1:1:0.9, by vol (10).

The remaining batch of large oysters and batch of small oysters were separately lyophilized prior to solvent extraction using a Javac SB9 (Knoxfield, Victoria, Australia) centrifugal freeze drier, model C.T. 1000. When completely dry (42 h), each sample was homogenized with a mortar and pestle and placed in the freeze drier for a further 2 h. A subsample of known weight (0.6 g dry weight) was taken and extracted with chloroform/methanol/water (1:2:0.8, by vol; 4 × 15 mL), ultrasonicated and centrifuged between extractions, until the last extract was colorless. Chloroform and water were added to the combined extracts from these lyophilized samples to give a final solvent ratio of chloroform/methanol/water (1:1:0.9, by vol) (10). At this stage, the same amount of water that had been removed from the tissues by lyophilization was added back in order to maintain this ratio.

The lower chloroform phase from each of the four samples (large and small "control" samples and large and small lyophilized samples) was reduced *in vacuo* to recover the lipids. These lipid extracts were then stored in chloroform under nitrogen at −20°C until further analysis.

Experiment 2. To verify the results from the first experiment, a further six fresh oysters (of larger size; mean soft tissue weight 9.8 ± 1.1 g) were harvested from the same location during the same season of the following year. The combined soft tissues of these oysters were frozen in liquid nitrogen, sliced finely, homogenized and the homogenate stored in liquid nitrogen prior to analysis. Six subsamples (1 g wet wt) of the homogenate were accurately weighed and four of these were lyophilized for 24 h. Two of the lyophilized subsamples were rehydrated prior to extraction by adding an amount of water equivalent to that removed by lyophilization, shaken and allowed to stand for 5 min. All samples, *i.e.*, the lyophilized duplicates, rehydrated-lyophilized duplicates and remaining "control" (nonlyophilized) duplicates were extracted according to the method outlined in Experiment 1 for lyophilized samples except that enough NaCl to make a 0.9% (wt/vol) solution was added to the aqueous phase to aid in phase separation (14,22).

Analyses of lipid classes and fatty acids. The lipid class compositions (Table 1) were determined by analyzing a portion of the total lipid extract with an Iatroscan Mk III TH-10 thin-layer chromatography/flame-ionization detector (TLC/FID) analyzer (Iatron Laboratories, Tokyo, Japan) as described by Volkman *et al.* (23), except that S-III silica rods were used and the TG standard curve was calibrated using a preparation of Atlantic salmon oil (99.4% TG). The solvent system used for the lipid class separation was hexane/diethyl ether/acetic acid (60:17:0.2, by vol), which separates most of the major nonpolar lipid classes found in oysters (23). Results (Table 1) are the average of three replicate rods (within ± 5% for each component), and Experiment 2 data are the average for duplicate extractions with standard deviation in parentheses showing the small variations associated with this method.

Aliquots of lipid extracts from Experiment 1 were transesterified to form fatty acid methyl esters (FAME) from esterified and free fatty acids (FFA) using methanol/chloroform/HCl (10:1:1, by vol; 3 mL) at 80°C for 2 h under high purity nitrogen. After cooling, 1 mL of water was added, and the FAME were extracted with hexane/chloroform (4:1, vol/vol; 3 × 3 mL). Another aliquot was saponified with 5% KOH (wt/vol) in methanol/water (4:1, vol/vol) at 80°C for 2 h. Sterols were extracted with hexane/chloroform (4:1, vol/vol) and derivatized with *bis*-(trimethylsilyl)trifluoroacetamide. FAME samples were analyzed with a Hewlett-Packard 5890 (Avondale, PA) GC equipped with an FID at a temperature of 280°C. FAME samples containing a known amount of methyl heptadecanoate (17:0 FAME) internal standard were injected using an air-cooled OCI-3 on-column injector (SGE, Ringwood, Victoria, Australia) onto a polar Supelcowax™ 10 fused-silica column (60 m × 0.32 mm i.d.; Supelco, Bellefonte, PA). The carrier gas was high purity hydrogen. Initially the GC oven was held at 45°C; 2 min after injection the temperature was increased at 30°C/min to 120°C then at 3°C/min to 250°C and held isothermal for 15 min. FAME and OTMS-sterols were analyzed using a Shimadzu GC-9A (Kyoto, Japan) GC also fitted with an air-cooled OCI-3 on-column injector, but with a nonpolar methylsilicone (HP-1) fused-silica capillary column (50 m × 0.32 mm i.d., Hewlett-Packard, Avondale, PA). GC conditions were identical to those of the polar column GC except that the FID temperature was 310°C, and the final oven temperature was held isothermal at 300°C for 25 min. Analyzing FAME on columns of two different polarities enabled identification and quantitation of coeluting components. Peak areas were quantified with Shimadzu CR-2A and CR-3A integrator/plotters.

Identification of fatty acids. Fatty acids were identified from retention index data on both polar and nonpolar columns and confirmed by interpretation of mass spectra

TABLE 1

Concentration (mg/g wet wt) of Lipid Classes and Percent Total Lipid (% wet wt and % dry wt) Extracted from "Control" (nonlyophilized), Lyophilized and Lyophilized Then Rehydrated Tissue of Different Size Classes of Adult *Crassostrea gigas*

	Lipid class (mg/g wet wt)[a]					Total lipid		Water
	PL	ST&DG	FFA	TG	HC&WE	(% wet wt)	(% dry wt)	content (%)
Experiment 1 (oyster size and lyophilization experiment)[b]								
"Control"—large	9.1	0.7	0.2	11.3	trace[c]	2.1	n.d.[d]	n.d.
"Control"—small	9.9	0.9	0.4	11.5	trace	2.3	10.2	77.6
Lyophilized (42 h)—large	9.8	1.2	0.4	0.9	0.1	1.2	6.4	80.6
Lyophilized (42 h)—small	8.6	1.0	0.1	0.7	trace	1.1	6.2	83.2
Experiment 2 (lyophilization and rehydration experiment)[e]								
"Control"	6.7	1.5	1.6	5.9	0.9	1.7	n.d.	n.d.
	(0.39)	(0.03)	(0.03)	(0.09)	(0.12)	(0.66)	n.d.	n.d.
Lyophilized (24 h)	6.6	1.5	1.4	3.1	0.6	1.3	7.2	81.7
	(0.21)	(0.01)	(<0.005)	(0.05)	(<0.005)	(0.02)	(0.09)	(<0.005)
Lyophilized (24 h) and rehydrated	6.5	1.4	1.6	5.4	0.8	1.6	8.2	80.9
	(0.11)	(0.01)	(0.03)	(0.26)	(0.03)	(0.04)	(0.34)	(0.34)

[a]PL, polar lipids; ST&DG, sterols plus diacylglycerols; FFA, free fatty acids; TG, triacylglycerols; HC&WE, hydrocarbons plus wax esters. Data based on average of triplicate analyses by TLC/FID, variation not exceeding ± 5% per component.
[b]Each data series is the analysis of a sample from the homogenate of six oysters of two size classes; large, average oyster soft tissue weight 8.9 ± 0.5 g; small, average oyster soft tissue weight 5.8 ± 0.4 g; "control"-solvent-extracted lipids from a nonlyophilized wet tissue sample; lyophilized-solvent extracted lipids from a freeze-dried sample; times (h) refer to duration of lyophilization.
[c]Trace = <0.1 mg/g wet wt.
[d]n.d., Not determined.
[e]Each data series is the average (SD in parentheses) of duplicate analyses of samples taken from the homogenate of six oysters of average soft tissue weight 9.8 ± 1.1 g; "control"-average solvent extracted lipids from nonlyophilized wet tissue samples; lyophilized-average solvent extracted lipids from freeze-dried samples; lyophilized and rehydrated-solvent extracted lipids from freeze-dried samples after rehydration with water for 5 min before extraction; times (h) refer to duration of lyophilization.

obtained with a Hewlett-Packard 5970B gas chromatography/mass spectrometry (GC/MS) system. GC/MS operating conditions were: electron multiplier, 2200 V; transfer line, 310°C; electron impact energy, 70 eV; 0.8 scan/s; mass range, 40–600 dalton. The dimethyldisulfide (DMDS) reaction (24) in conjunction with GC/MS was used to identify monoenoic fatty acid double-bond positions and geometries.

Fatty acid identifications were confirmed by argentation TLC using cleaned Macherey-Nagel (Düren, Germany) SIL G-100 UV_{254} TLC plates which had been impregnated with silver nitrate. Methyl esters were separated by double development in a single solvent system (hexane/diethyl ether/acetic acid, 94:4:2, by vol; Ref. 25). After development, each band (containing FAME of the same degree of unsaturation) was scraped from the plates, extracted and analyzed on polar and nonpolar capillary columns by GC and GC/MS. This enabled the degree of unsaturation of each fatty acid and the geometry of the double bond in *trans*-16:1n-10 to be determined, since the *trans*-monoenoic FAME characteristically elute in the band between the saturated and *cis*-monoenoic fatty acids. The unusual odd chain fatty acid 21:5n-3 eluted with other pentaenoic FAME. The nonmethylene interrupted dienoic (NMID) fatty acids eluted between the methylene interrupted dienoic and the trienoic fatty acids, the nonmethylene interrupted trienoic (NMIT) fatty acid co-eluted with the methylene interrupted trienoic fatty acids (the separation between trienoic and tetraenoic methylene interrupted fatty acids was insufficient to permit analysis of a band between them).

Samples of oyster FAME and FAME standards (Nu-Chek-Prep Inc., Elysian, MN) were analyzed to determine the double-bond positions in all PUFA including verification of the NMID and NMIT fatty acids using a modification of Fay and Richli's (21) procedure for the formation of DMOX derivatives. Solvent was removed from the FAME under nitrogen; they were then reacted with 500 μL of 2-amino-2-methylpropanol under nitrogen overnight at 140°C instead of 180°C (to reduce the loss of unsaturated fatty acids). To reduce time, solvent usage and loss of sample, smaller volumes of washing and extraction solvent were used according to the following method. After the 2.5 mL vials containing the samples were allowed to cool, 1 mL of dichloromethane and 1 mL of water were added. After shaking and allowing the phases to settle, the upper aqueous layer was discarded, and each sample was washed a further three times prior to being dried with anhydrous sodium sulfate. The solvent in each vial was transferred to a clean vial and evaporated under nitrogen. The residue consisting of DMOX derivatives of FAME was made up in chloroform and analyzed by GC/MS.

RESULTS AND DISCUSSION

Lipid classes extracted from C. gigas *and the effect of lyophilization on composition.* The solvent extracted lipids of the "control" (nonlyophilized) samples of *C. gigas* were composed mostly of PL and TG with low contents of sterols and FFA (Table 1). These "control" samples provide accurate data against which results for lipid extracts from lyophilized samples could be compared. There was no

obvious difference between the lipid classes extracted from oysters of different sizes (large *cf.* small, Experiment 1).

Extracts from samples which had been lyophilized prior to analysis had similar contents of polar lipids, sterols and FFA to extracts from "control" samples, but there were markedly reduced levels of triacylglycerols extracted (Table 1). There was no increase in the amount of other lipid classes so the loss of TG was not due to degradation to form mono- and diacylglycerols (DG) and FFA since these would have been detected (Table 1). In the first experiment only 7% of the TG were extracted compared with the nonlyophilized "control" samples. In Experiment 2, 52.5% of the TG were extracted relative to the "control" samples. The reasons for the different extents of extraction are unclear, although the higher initial contents of TG in the oysters of Experiment 1 compared to those in the second experiment may have been a factor. Also, because of different sample weights, oyster samples from Experiment 1 were lyophilized for longer than samples in Experiment 2 (42 *vs.* 24 h). A further experiment showed that there was no further decrease in the efficiency of extraction of the TG after 8 h in the freeze drier, with the same amount of TG being extracted from samples lyophilized for 8, 16 and 24 h (Dunstan, G.A., and Volkman, J.K., unpublished data). Perhaps more significantly, the samples in Experiment 1 were lyophilized prior to homogenization, whereas in Experiment 2 the samples were homogenized first.

Rehydration prior to solvent extraction, as recommended by Christie (15), significantly increased the amount of TG extracted from lyophilized samples (Table 1). Therefore if lyophilization must be used prior to solvent extraction of lipids from adult oysters, it is recommended that the tissues be rehydrated prior to extraction. The original schemes of Holland and Gabbott (18) and Mann and Gallager (26) incorporate rehydration of the sample prior to analysis, whereas the scheme of Whyte *et al.* (20) does not.

The reasons for the reduced extraction of TG from lyophilized tissues are unclear, although Nelson (14) has suggested that extraction of hydrophobically bound lipids from lyophilized tissues is more difficult because proteins provide an ionic barrier to nonpolar solvents. However, it should be noted that the Bligh and Dyer (10) solvent system is a mixture of both polar and nonpolar solvents. Reduced extraction of lipids from freeze-dried samples may be associated with the high concentration of glycogen in molluscs (Ackman, R.G., personal communication), or due to lyophilization possibly altering the solubility of macromolecules (22).

Significant reductions in the amounts of *n*-alkanes, cycloalkanes and polyaromatic hydrocarbons as well as reduced recoveries of added internal standards from lyophilized mussel homogenate relative to nonlyophilized homogenate have been demonstrated by Farrington *et al.* (27). They suggested that the losses may have been due in part to volatilization, but also possibly to removal of some hydrocarbons from the extraction solution by absorption or adsorption. Interestingly, the greatest losses of *n*-alkanes were between *n*-C_{19} and *n*-C_{25}; lighter and heavier molecules were relatively unaffected. Other potential problems associated with lyophilization are the formation of artifacts, particularly in sediment samples (17), and loss of organic molecules of low to intermediate molecular weight due to the high vacuum of a freeze drier (16).

Fatty acids extracted from C. gigas *and the effect of lyophilization on composition.* Sixty-four fatty acids were identified in the samples of *C. gigas* analyzed. The fatty acid compositions of the "control" group (Table 2) are typical for adult oysters of *C. gigas* (6) and provide accurate data against which results for lipid extracts from lyophilized samples can be compared. All samples had high proportions of PUFA (51.1–55.5% of total fatty acids), the most abundant being 20:5n-3 (15.3–17.0%) and 22:6n-3 (20.2–25.0%), which is typical of most marine invertebrates (28). In *C. virginica* the proportions of these long-chain n-3 PUFA vary seasonally with diet and reproduction (29). There was very little difference between the amounts and distribution of fatty acids in oysters of different sizes (Table 2).

The major saturated fatty acid in all samples of *C. gigas* was 16:0 (15.4–18.6%), and the most abundant monoenoic fatty acids were 18:1n-9 (2.4–4.5%), 18:1n-7 (2.8–3.4%) and 20:1n-7 (2.7–3.9%; Table 2). The saturated and monoenoic fatty acids present in this species are partly of dietary origin although they may also be formed *de novo* by the animal (30). Small contents of branched-chain (mostly *iso*- and *anteiso*-) fatty acids (2.3–3.0%) were detected in *C. gigas*, with little difference between extracts from "control" and lyophilized samples (Table 2). Although the main source of monomethyl branched-chain fatty acids is probably bacteria in the gut and tissues of the animal, some of these fatty acids may also be normal minor metabolites derived from amino acid precursors (30). The isoprenoid fatty acids 4,8,12 TMTD (trimethyltridecanoic acid), pristanic and phytanic acids are derived from the isoprenoid moiety (phytol) in chlorophylls present in the microalgal diet (30).

The fatty acid composition of the TG generally reflects the animal's diet (because TG are the major storage lipid for dietary fatty acids as well as those synthesized *de novo*), whereas the PL fatty acid composition is selectively modified by the oyster according to cellular membrane requirements (2,9). Relative to the PL, the TG generally contain higher proportions of 16:0 and C_{18} unsaturated fatty acids but lower proportions of C_{20} and C_{22} unsaturated fatty acids (2,8). Extracts from lyophilized samples had very different fatty acid distributions relative to the extracts from "control" samples with less 16:0 and C_{18} unsaturated fatty acids resulting in artificially high proportions of C_{20} and C_{22} unsaturated fatty acids (Table 2). These variations in fatty acid distributions in oysters from different treatments were probably the result of the reduced extraction of TG from lyophilized samples. Ackman (30) has suggested that dietary 18:4n-3 (stored in the TG) after chain elongation is desaturated by a Δ5 desaturase to form 20:5n-3 and incorporated into the PL, explaining the reduced contents of 18:4n-3 in extracts from lyophilized samples.

Although the importance of dietary long-chain PUFA is relatively well established, the functions of the unusual odd-chain 21:5n-3 is unclear. This PUFA has been found in molluscs, rotifers, fish and marine mammals (6,31,32). Ackman (32) has proposed that 21:5n-3 is formed by α-oxidation of 22:5n-3, and it may be that this system is common in invertebrates.

Many authors have used lyophilization prior to lipid

TABLE 2

Fatty Acid Composition (% of total fatty acids) of the Lipid Extracted from Nonlyophilized or Wet Tissue ("Control") and from Lyophilized Tissue of Two Size Classes of Adult *Crassostrea gigas*

	"Control"[a]		Lyophilized[b]	
Fatty acids	Large[c]	Small[c]	Large	Small
Saturated				
14:0	2.3	2.3	1.4	1.4
15:0	0.8	0.8	0.6	0.7
16:0	18.2	18.6	15.6	15.4
18:0	3.4	3.5	4.0	3.7
19:0	0.1	0.1	0.1	0.1
20:0	0.1	0.1	0.1	0.1
22:0	0.1	0.1	0.1	0.1
24:0	0.2	0.1	0.1	0.1
Subtotal[d]	25.2	25.6	21.9	21.5
Branched-chain				
4,8,12 TMTD	0.5	0.6	0.8	1.0
*i*15:0	0.2	0.2	0.2	0.1
*i*16:0	0.2	0.2	0.1	0.2
*i*17:0	0.5	0.5	0.5	0.4
*a*17:0	0.1	0.1	0.2	0.2
*br*18:0	0.4	0.4	0.4	0.4
*i*18:0	0.1	0.1	0.1	0.2
phytanic acid	0.1	0.1	0.1	0.1
*a*19:0	0.2	0.2	0.3	0.3
Subtotal[d]	2.3	2.4	2.8	3.0
Monounsaturated				
16:1n-12	0.2	0.2	0.2	0.1
16:1n-11	0.1	0.1	0.1	trace[e]
16:1n-10	0.5	0.8	0.3	0.4
16:1n-9	0.1	0.2	0.1	0.1
16:1n-7	1.4	1.1	0.9	0.9
16:1n-5	0.3	0.3	0.2	0.3
17:1n-8	0.3	0.2	0.2	0.2
18:1n-13	0.8	0.9	0.4	0.2
18:1n-9	4.5	4.4	2.8	2.4
18:1n-7	3.4	3.4	2.8	2.8
18:1n-5	0.1	0.2	0.2	0.2
20:1n-15	0.1	0.1	trace	0.1
20:1n-14	0.1	0.3	0.2	0.2
20:1n-13	1.1	1.0	1.0	1.1
20:1n-9	0.6	0.8	1.3	1.2
20:1n-7	2.7	2.7	3.9	3.7
Subtotal[d]	15.6	15.8	14.0	13.2
Polyunsaturated (PUFA)				
18:2n-6	2.0	2.0	1.3	1.0
18:3n-6	0.2	0.2	0.1	0.1
18:3n-3	2.1	2.1	1.5	1.1
18:4n-3	4.3	4.6	3.5	2.9
20:2n-6	0.3	0.3	0.3	0.3
20:3n-6	0.2	0.2	0.1	0.1
20:3n-3	0.2	0.2	0.1	0.1
20:4n-6	2.6	2.4	3.4	3.9
20:4n-3	0.7	0.8	0.7	0.6
20:5n-3	15.7	15.3	15.9	17.0
21:5n-3	0.8	0.7	0.6	0.6
22:4n-6	0.2	0.2	0.3	0.3
22:5n-6	0.5	0.5	0.6	0.6
22:5n-3	1.3	1.2	1.4	1.4
22:6n-3	20.7	20.2	24.3	25.0
Subtotal[d]	51.8	51.1	54.4	55.5
Nonmethylene interrupted (NMI)				
20:2(*5,11*)	0.2	0.2	0.1	0.1
20:2(*5,13*)	0.6	0.7	0.3	0.3
22:2(*7,13*)	0.9	0.9	1.3	1.4
22:2(*7,15*)	2.6	2.5	3.4	3.7
22:3(*7,13,16*)	0.3	0.2	0.4	0.5
Subtotal	4.5	4.5	5.5	5.9
Not identified	0.6	0.7	1.3	1.0
Total	100.0	100.0	100.0	100.0

[a]"Control," solvent-extracted lipids from a nonlyophilized wet tissue sample.
[b]Lyophilized, solvent-extracted lipids from a freeze-dried sample.
[c]Large, average oyster soft tissue weight 8.9 ± 0.5 g; small, average oyster soft tissue weight 5.8 ± 0.4 g.
[d]Subtotals include contributions from minor fatty acids 21:0, 23:0, *i*14:0, *a*15:0, pristanic acid, *br*17:0, 22:1, 24:1, 16:2n-4, 18:2n-9?, 18:2n-3.
[e]Trace = <0.05%.

analysis (33,34), but very few direct comparisons have been made with solvent extraction of fresh tissue. Although such methods are fast and convenient, our data suggest that direct comparisons with solvent extraction of fresh or frozen samples should be performed to validate data obtained from lyophilized samples. In our experience, lyophilizaton does not affect the solvent extraction of lipids from fish flesh and prawn larvae (Dunstan, G.A., and Volkman, J.K., unpublished data). Interestingly, others have found little difference in the FAME distribution of saponified then methylated lyophilized oyster and scallop eggs and larvae, a diatom, rotifers and certain fish tissues compared with the FAME distribution of solvent extracted lipids from lyophilized samples of the same tissues (31). However, significant differences between FAME distributions of directly saponified then methylated and solvent extracted lipids from lyophilized fish faeces have been noted (35), whereas in the same study such differences were not observed in analyses of nonlyophilized fish diets. Lyophilization apparently did not affect the amount of lipid extracted from adult *C. madrasensis*, although lipid determinations were made on extracts after acid hydrolysis and not solvent extraction of native tissues (36).

It is not only lyophilization which reduces solvent extraction of lipids from certain samples. For example, we have observed that solvent extraction of lipids from steam-pelleted fish feeds was also significantly reduced (Dunstan, G.A., and Volkman, J.K., unpublished data). Rehydration did not increase extraction efficiency from these dried samples, but increasing the solvent contact time greatly increased the yield. Reduced extraction of lipids by a modified Bligh and Dyer method has also been observed with vacuum or hot air dried (but not lyophilized) brine shrimp *Artemia* (37).

Most comparative work has been performed with microalgae, but the results are often divergent making it difficult to draw significant conclusions. Guckert *et al.* (13) showed that Soxhlet extraction significantly increases the extraction of neutral lipids relative to Bligh and Dyer solvent extraction from lyophilized cells of the green microalga *Chlorella*. These authors suggested that this increase was due to transesterification of fatty acids even though there was no corresponding decrease in the PL classes from which these FAME were presumably derived. Alternatively, Soxhlet extraction may free the fatty acids from those TG which were not extracted by the Bligh and Dyer solvent system. However, the possible loss of the more labile PUFA during prolonged Soxhlet extraction also needs to be considered (13).

NMI fatty acids in C. gigas. The NMI FAME were tentatively identified based on their mass spectra. The mass spectra of NMID resembled those of monoenoic methylene interrupted fatty acids, with a base peak at *m/z* 55. Other major ions were *m/z* 67 and *m/z* 81 both of which are indicative of dienoic methylene interrupted fatty acids (*e.g.*, Fig. 1). The mass spectra of the C_{20} and C_{22} NMID had molecular ions at *m/z* 322 and *m/z* 350 (Fig. 1) respectively, establishing the presence of two double bonds. The mass spectrum of the NMIT resembled that of a dienoic methylene interrupted fatty acid (due to the methylene interrupted double bonds), with a base peak at *m/z* 67 and strong *m/z* 81, other major ions were *m/z* 79 and *m/z* 91,

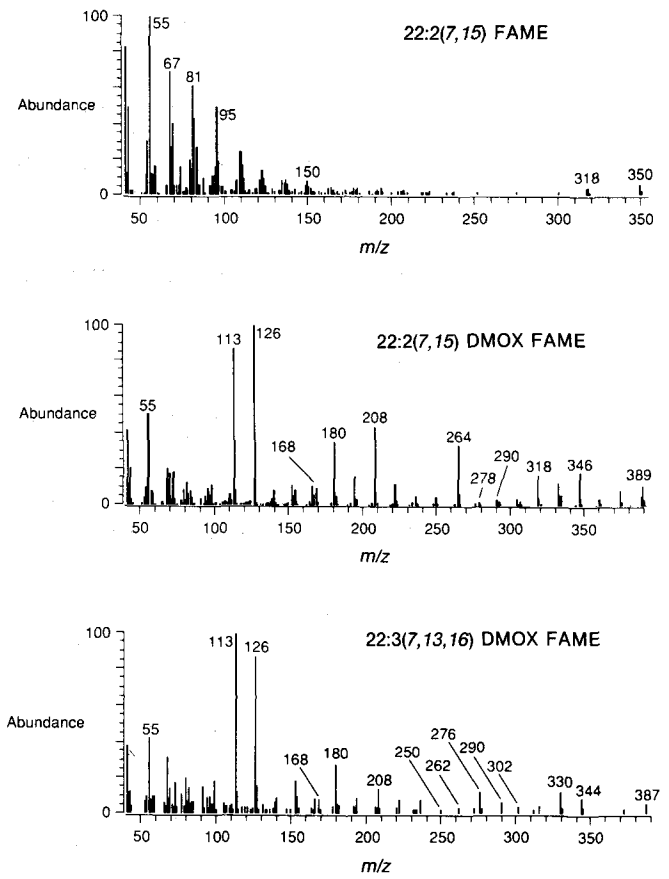

FIG. 1. Selected mass spectra of nonmethylene interrupted fatty acid methyl esters (FAME) from *Crassostrea gigas*. Top, mass spectrum of 22:2(*7,15*) nonmethylene interrupted diene (NMID) FAME, with a base peak at *m/z* 55, other major ions were *m/z* 67 and *m/z* 81 and molecular ion of *m/z* 350. Middle, mass spectrum of 22:2(*7,15*) NMID FAME 4,4-dimethyloxazoline (DMOX) derivative with 12 dalton between *m/z* 168 and *m/z* 180 (double bond at Δ7) and between *m/z* 278 and *m/z* 290 (double bond at Δ15) and a molecular ion at *m/z* 389 (C_{22} FAME DMOX derivative with two double bonds). Bottom, mass spectrum of 22:3(*7,13,16*) NMIT FAME DMOX derivative with 12 dalton between *m/z* 168 and *m/z* 180 (double bond at Δ7), *m/z* 250 and *m/z* 262 (double bond at Δ13) and *m/z* 290 and *m/z* 302 (double bond at Δ16) and a molecular ion at *m/z* 387 (C_{22} FAME DMOX derivative with three double bonds).

which are indicative of three or more double bonds, but the M^+ was not detected in the mass spectrum of the C_{22} NMIT FAME.

The modified method of DMOX derivatization resulted in reduced loss of PUFA, while still ensuring complete reaction of the FAME. Double-bond positions were readily identified according to the criteria outlined in Fay and Richli (21) with distinctive ion abundance patterns being evident for monoenoic, polyenoic FAME, terminal ω end double bonds and NMI and conjugated PUFA. Figure 1B shows the mass spectrum of the DMOX derivative of the NMID FAME 22:2(*7,15*) with 12 dalton between *m/z* 168 and *m/z* 180 (Δ7) and between *m/z* 278 and *m/z* 290 (Δ15) and a molecular ion at *m/z* 389 establishing the presence of two double bonds in a C_{22} FAME DMOX derivative. Figure 1C shows the mass spectrum of the DMOX derivative of the NMIT FAME 22:3(*7,13,16*) with 12 dalton between *m/z* 168 and *m/z* 180 (Δ7), *m/z* 250 and *m/z* 262 (Δ13) and *m/z* 290 and *m/z* 302 (Δ16) and a molecular ion at *m/z* 387 clearly establishing the presence of three double bonds in the C_{22} FAME DMOX derivative. These fragments are the same as those for the equivalent bond positions using pyrrolidine derivatization (38). The DMOX derivatization of FAME enabled positive identification of the double-bond positions in the 21:5n-3, NMID, NMIT and the more common unsaturated FAME.

Organisms known to contain NMI fatty acids include species of bacteria, macroalgae and marine invertebrates (39; reviewed in Refs. 28,32). The presence of both 18:1n-13 and 20:1n-15 suggested the existence of a Δ5 desaturase in those molluscs which produce NMID fatty acids (40). In the present study these monoenoic fatty acids as well as 16:1n-11 were detected in small amounts (<1%, Table 2). Ackman and Hooper (40) proposed a biosynthetic pathway for the formation of NMID in molluscs which involves a Δ5 desaturase acting on 20:1n-7 and 20:1n-9 to form 20:2(*5,13*) and 20:2(*5,11*), respectively. Chain elongation of these two C_{20} NMID produces 22:2(*7,15*) and 22:2(*7,13*), respectively. The presence and abundance of these isomers suggests that the same or a similar pathway is acting in *C. gigas* (Table 2). The synthesis of these NMID *via* these pathways in bivalve molluscs has been verified using [^{14}C]acetate (41, and references therein), although it is not known whether bacteria present in the tissues of *C. gigas* play a role in NMI fatty acid formation by Δ5 desaturation. The 22:3(*7,13,16*) NMIT is most likely the result of the Δ5 desaturation and subsequent elongation of 20:2n-6. Although a candidate for the intermediate C_{20} trienoic NMI was detected, its abundance was too low for positive identification. Interestingly, desaturation of the 22:3(*7,13,16*) NMIT at the Δ10 carbon would yield 22:4n-6 which is the shortest methylene interrupted C_{22} PUFA detected in *C. gigas* (Table 2). The NMIT fatty acid 22:3(*7,13,16*) was originally identified from a sponge extract (38), but to our knowledge has not been recorded in oysters, although the unusual NMIT 22:3(*7,11,15*) was recently detected in hydrocarbon seep mussels (42). In *C. virginica* the 22:2 NMI fatty acids are abundant in PL (8,39), whereas the 20:2 NMI are primarily resident in the TG (8). This possibly explains the enhanced proportions of 22:2 NMID fatty acids and reduced 20:2 NMID fatty acids in the extracts from lyophilized samples of *C. gigas* with reduced contents of TG (Table 2).

The effect of lyophilization on sterols. C. gigas contained a complex sterol distribution with no significant difference between the two size classes (Table 3). However, extracts from lyophilized samples had significantly lower amounts of 24-ethylcholest-5-en-3β-ol (0.8 and 0.9%) compared with extracts from "control" samples (6.0 and 5.7%; Table 3), implying that lyophilization also affects the extraction efficiency of this sterol. Most of the sterols listed in Table 3 were probably derived from a diet rich in microalgae although 24-ethylcholest-5-en-3β-ol can also be indicative of terrestrial plant matter and sea-grasses, and it is common in marine sediments (43). Data on the ability of bivalve molluscs to synthesize sterols *de novo* is often contradictory, but these molluscs have the ability to form cholesterol from dietary phytosterols, albeit at a reduced level (44).

TABLE 3

Sterol Composition (% of total sterols) and Total Sterol Content (mg/g wet wt and mg/g dry wt) of the Lipid Extracted from Nonlyophilized or Wet Tissue ("Control") and from Lyophilized Tissue of Two Size Classes of Adult *Crassostrea gigas*

Sterol	Trivial name	"Control"[a]		Lyophilized[b]	
		Large[c]	Small[c]	Large	Small
24-Norcholesta-5,22-dien-3β-ol		5.1	5.4	4.9	5.2
27-Nor-24-methylcholesta-5,22E-dien-3β-ol	Occelasterol	4.9	5.1	4.9	5.0
di-Cholesta-5,22E-di-en-3β-ol	*trans*-22-Dehydrocholesterol	12.2	12.7	11.6	12.3
5α-Cholest-22E-en-3β-ol	*trans*-22-Dehydrocholestanol	0.6	0.6	0.5	0.6
Cholest-5-en-3β-ol	Cholesterol	28.6	26.7	27.1	27.6
5α-Cholestan-3β-ol	Cholestanol	2.2	2.0	1.9	2.0
Cholesta-5,24-dien-3β-ol	Desmosterol	0.5	1.5	1.5	2.3
24-Methylcholesta-5,22E-dien-3β-ol	Brassicasterol/Crinosterol	13.5	14.8	16.8	15.7
24-Methylcholesta-5,24(28)-dien-3β-ol	24-Methylenecholesterol	12.2	12.0	13.8	13.4
24-Methylcholest-5-en-3β-ol	Campesterol/Dihydrobrassicasterol	1.7	1.7	1.8	1.8
24-Ethylcholesta-5,22E-dien-3β-ol	Stigmasterol/Poriferasterol	1.9	1.3	2.1	2.0
24-Ethylcholest-5-en-3β-ol	Sitosterol/Clionosterol	6.0	5.7	0.8	0.9
24-Ethylcholesta-5,24(28)E-dien-3β-ol	Fucosterol	1.1	1.2	1.3	1.1
24-Ethylcholesta-5,24(28)Z-dien-3β-ol	Isofucosterol	7.9	7.8	9.1	8.3
24-Propylcholesta-5,24(28)-dien-3β-ol	24-Propylidenecholesterol	1.5	1.4	1.9	1.7
Total sterols					
Concentration (mg/g wet wt)		1.2	1.2	1.1	0.9
Concentration (mg/g dry wt)		n.d.[d]	5.4	5.5	5.6

[a]"Control," solvent-extracted lipids from a nonlyophilized wet tissue sample.
[b]Lyophilized, solvent-extracted lipids from a freeze-dried sample.
[c]Large, average oyster soft tissue weight 8.9 ± 0.5 g; small, average oyster soft tissue weight 5.8 ± 0.4 g.
[d]n.d., Not determined.

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METHOD

Rapid Separation of the Major Phospholipid Classes on a Single Aminopropyl Cartridge

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A rapid method for the separation of the individual phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) by a single solid-phase extraction was developed. PC, PE, PS and PI were sequentially eluted from aminopropyl bonded silica with acetonitrile/*n*-propanol (2:1, vol/vol), methanol, isopropanol/methanolic HCl (4:1, vol/vol) and methanol/methanolic HCl (9:1, vol/vol). Standard recoveries were over 95% for PC and PE and over 85% for PS and PI with undistorted fatty acid composition. The separation of complex lipid mixtures on aminopropyl minicolumns can be refined to the level of individual phospholipid classes.
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The rapid group separation of complex lipid mixtures by solid-phase extraction on an aminopropyl bonded phase was introduced by Kaluzny *et al.* (1) in 1985. This convenient method was extended by Kim and Salem (2) in 1990 to allow the separation of neutral from acidic phospholipids (PL) using an additional acidic eluent. The separation of individual PL classes, however, still required thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (3), which are much more laborious and have several drawbacks. We therefore developed a new system of eluents to further refine the separation of lipids on a single aminopropyl bonded minicolumn to the further level of individual PL classes.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC), egg yolk phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), soybean phosphatidylinositol (PI) (all of >98% purity) and *all-cis*-eicosadienoic acid (20:2n-6) were from Sigma (St. Louis, MO). 1,2-Di-[1′-^{14}C]palmitoyl PC (112 mCi/mmol), 1-palmitoyl-2-[(1′-^{14}C]linoleoyl PE (53 mCi/mmol), 1,2-dioleoyl phosphatidyl-[3′-^{14}C]serine (53 mCi/mmol), 1,2-diacyl-*sn*-glycero-3-phospho-[2′-^{3}H]inositol (18.2 Ci/mmol) and (*N-methyl*-^{14}C)sphingomyelin (SM; 52 mCi/mmol) prepared from bovine brain were from Amersham (Little Chalfont, Amersham, United Kingdom). Butylated hydroxytoluene (BHT) was obtained from Serva (New York, NY). Aminopropyl bonded silica minicolumns (100 and 500 mg) and silica minicolumns (100 mg) were from J.T. Baker (Deventer, The Netherlands). Water-free 3N methanolic HCl was from Supelco (Bellefonte, PA). All other solvents (analytical grade), molybdatophosphoric acid hydrate and silica G60 plates for TLC were purchased from Merck AG (Darmstadt, Germany). A "Vac Elut" apparatus from Analytichem International (Harbour City, CA) was used for flushing the cartridges. Screw-capped glass reaction vials were from Schott (Jena, Germany). Plasma lipid extracts were prepared by the method of Folch *et al.* (4) in the presence of BHT.

TLC. PL classes were separated on G60 silica plates developed in chloroform/methanol/acetic acid/water (100:75:7:4, by vol) (5). For visualization of the lipid fractions, the plates were sprayed with a 10% solution of molybdatophosphoric acid hydrate in ethanol and heated at 120°C for 5 min. Approximate R_f values in this systems are 0.7 for PE, 0.4 for PI, 0.3 for PS and 0.15 for PC.

Solid-phase extraction procedure. The 100-mg (500-mg) aminopropyl cartridges were preconditioned with 1 mL (3 mL) hexane. Then the sample was applied to the 100-mg (500-mg) cartridge in 100 μL (300 μL) chloroform and left to sink into the solid phase. Solvents were then completely sucked through the solid phase under slight vacuum, but then the vacuum was released to avoid prolonged air passage and drying of the solid phase. The sequence of solvents and the optimized solvent volumes used for 100-mg and 500-mg cartridges, respectively, are listed in Table 1.

First, the neutral lipids and then the free fatty acids were eluted with chloroform/isopropanol (2:1, vol/vol) and diethyl ether/acetic acid (98:2, vol/vol), respectively. These

TABLE 1

Sequence of Eluents, Eluted Lipids, Solvents and Eluent Volumes Used for 100-mg and 500-mg Aminopropyl Cartridges

Eluent	Eluted lipid[a]	Solvents	Eluent volumes (mL) 100-mg[b]	500-mg[b]
1	NL	Chloroform/isopropanol (2:1, vol/vol)	1.2	4
2	FFA	Diethyl ether/acetic acid (98:2, vol/vol)	1.2	4
3, A	PC	Acetonitrile/*n*-propanol (2:1, vol/vol)	2.4	9
4, B	PE	Methanol	1.2	3
5, C	PS	Isopropanol/methanolic HCl (4:1, vol/vol)	0.9	4.5
6, D	PI	Methanol/methanolic HCl (9:1, vol/vol)	1.2	6

[a]Abbreviations: NL, neutral lipids; FFA, free fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.
[b]Sorbent amount of the cartridges.

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Abbreviations: BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography.

first two steps were done with all samples as described by Kaluzny *et al.* (1). Then individual PL classes were sequentially eluted using our newly developed solvent systems as follows: PC was eluted with acetonitrile/*n*-propanol (2:1, vol/vol) (Solvent 3, A), PE was eluted with methanol (Solvent 4, B), then PS was eluted with isopropanol/methanolic HCl (4:1, vol/vol) (Solvent 5, C) and finally PI was eluted with methanol/methanolic HCl (9:1, vol/vol) (Solvent 6, D).

Quantification. Aliquots of about 5000 cpm of each of the phospholipids were either directly transferred to a scintillation vial or applied to aminopropyl cartridges. Each fraction eluted from the cartridge was transferred to a scintillation vial, brought to dryness under nitrogen and taken up again in 0.5 mL ethanol. All vials were filled with 5 mL "Aquasafe 300" scintillation solution (Zinsser Analytik, Frankfurt, Germany) and counted for 2 min on a Beckman LS 5801 liquid scintillation system (Munich, Germany). Recoveries in each fraction are expressed as percentage of the total counts applied to the cartridge.

Transmethylation and gas chromatography. Samples were taken up in 1 mL of methanol and 2 mL of methanolic HCl (3N). Forty μg of 20:2n-6 was added as internal standard. The vials were sealed, heated to 90°C for one hour and then cooled in water. After adding 2 mL of distilled water the fatty acid methyl esters were extracted twice with 2 mL of petroleum hydrocarbon (b.p. 40–60°C), dried and stored in 40 μL hexane. A 4 μL aliquot of the sample was injected onto a Hewlett-Packard 5890 gas chromatograph equipped with a capillary column of 0.25 mm i.d. and 30-m length coated with DB 225 0.15μ from ICT (Frankfurt, Germany). The injector temperature was set at 200°C, helium flow was 1 mL/min and the column temperature was linearly increased from 90°C at the start at 70°C/min and then held at 200°C. The detector temperature was 250°C.

RESULTS AND DISCUSSION

Kim and Salem (2) had reported partial resolution of PL on an aminopropyl cartridge by eluting PC and PE with methanol, and PS and PI with hexane/isopropanol/ethanol/0.1 M ammonium acetate in water/formic acid (420:350:100:50:0.5, by vol) with 5 mL phosphoric acid added per 100 mL. We tried to further refine the resolution to the level of individual PL classes. First, a series of solvents of increasing polarity was tested for differential PL elution of PC and PE from the aminopropyl matrix. Acetonitrile partially eluted PC, but not PE. *n*-Propanol and all solvents more polar than acetonitrile eluted both PC and PE, whereas solvents less polar than acetonitrile eluted neither compound. A mixture of acetonitrile and *n*-propanol (2:1, vol/vol) quantitatively eluted PC, whereas PE was completely retained. PE could subsequently be eluted with methanol (Fig. 1).

Second, methanolic HCl was found to be the simplest acidic solvent to elute both PS and PI from the aminopropyl matrix. Therefore, mixtures of less polar solvents with methanolic HCl were tested for differential elution of PS and PI. Isopropanol/methanolic HCl (4:1, vol/vol) was found to elute PS, whereas PI was retained. PI could subsequently be eluted with methanol/methanolic HCl (9:1, vol/vol) (Fig. 1).

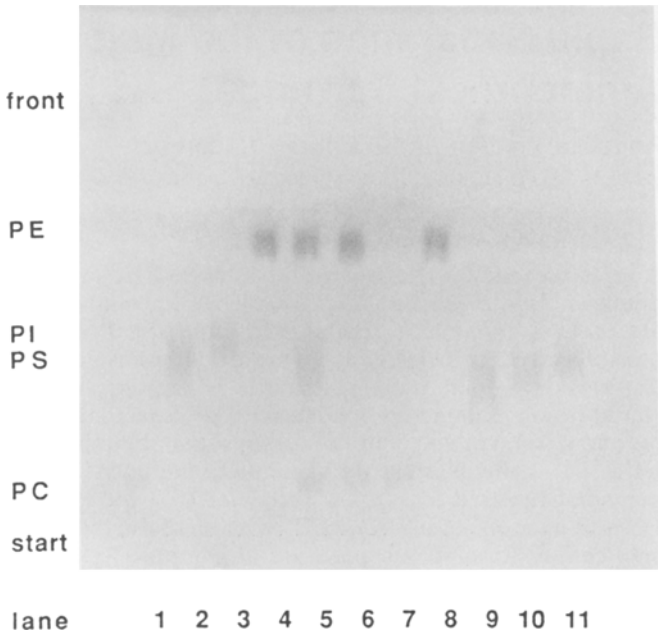

FIG. 1. Verification by thin-layer chromatography (TLC) of the separation of lipid mixtures by sequential elution from a single aminopropyl minicolumn. TLC and staining was as described in the Materials and Methods section. Lanes 1 to 4: reference standards of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidyinositol (PI) and phosphatidylethanolamine (PE). Lane 5: Standard mixture as applied to the minicolumn. Lane 6: Phospholipids fraction eluted according to Reference 1 contains PC and PE. Lane 7: Eluent A contains PC only. Lane 8: Eluent B contains PE. Lane 9: Acidic eluent according to Reference 2 contains PS and PI. Lane 10: Eluent C contains only PS. Lane 11: Eluent D contains PI.

Labeled standards were used to confirm and to optimize the elution volumes for cartridges filled with 100 and 500 mg sorbent, and the results obtained were verified by TLC. The eluents and volumes established for the definitive procedure are listed in Table 1. Recoveries in each fraction for pure standards and standards added to plasma extracts were determined for each PL class. The results are summarized in Table 2.

Changes in fatty acid composition due to preferential retention of specific molecular species during the separation procedure could be excluded. Relative amounts of fatty acid methyl esters prepared from each PL standard were found unchanged after the samples had been carried through the separation procedure twice (Table 3).

With 100-mg cartridges, the binding and separation of labeled standard PL was unchanged by the presence of cold PL in the mass range from 1 to 500 μg. At higher concentrations PC was not completely retained and was partially eluted in the neutral lipid fraction.

Two other PL classes, SM and phosphatidic acid (PA), can be present in biological samples. SM was eluted with acetonitrile/*n*-propanol (2:1, vol/vol), and PA was eluted with isopropanol/methanolic HCl (4:1, vol/vol). Attempts to separate SM from PC and to separate PA from PS on the same cartridge were not successful. The contamination of PC with SM and PS with PA is even a problem with sophisticated gradient HPLC methods (6) and should not compromise this method for many applications. SM will not interfere with fatty acid analyses of PC, as the

TABLE 2

Recoveries and Relative Purities of Labeled PL[a] Standards[b]

% Recovery[c]	Fraction A	B	C	D
Standard (100-mg[d])				
PC	96.4 ± 1.1	4.1 ± 1.1	0.5 ± 0.4	0.4 ± 0.4
PE	0.0 ± 0.1	101.5 ± 1.4	1.0 ± 0.3	0.7 ± 0.3
PS	0.3 ± 0.1	0.3 ± 0.1	88.5 ± 1.6	8.7 ± 0.7
PI	0.1 ± 0.1	0.3 ± 0.2	9.5 ± 1.6	86.2 ± 2.5
Standard (500-mg[e])				
PC	96.9 ± 1.8	2.9 ± 0.5	—[f]	0.1 ± 0.0
PE	0.1 ± 0.2	99.7 ± 3.6	—	0.3 ± 0.0
PS	—	0.1 ± 0.0	91.5 ± 3.4	5.9 ± 1.0
PI	—	0.5 ± 0.6	9.5 ± 1.5	81.9 ± 1.5
Standard added to plasma (100-mg[g])				
PC	85.9	4.9	1.3	9.9
PE	0.6	99.8	2.0	3.4
PS	0.1	0.2	84.0	6.8
PI	—	0.4	9.9	81.6

[a]Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.
[b]Pure labeled PL standards (n = 5) or labeled standards added to plasma (in duplicate) in fractions A, B, C and D eluted from 100-mg cartridges and 500-mg cartridges.
[c]The data represented are the means ± SD for standards, n = 5; means of duplicate for plasma with respect to reference radioactivity measured for a nonextracted duplicate.
[d]Labeled PL standards isolated and purified on a 100-mg aminopropyl cartridge.
[e]Labeled PL standards isolated and purified on a 500-mg aminopropyl cartridge.
[f]Less than detection limit of about 0.1%.
[g]Recovery of PL standards added to plasma extracts purified on 100-mg cartridges.

TABLE 3

Fatty Acid Composition of PL[a] Standards Before (1) and After (2) Repetitive Extractions on Aminopropyl Bonded Silica[b]

Fatty acid[c] (%)	PC (1)	PC (2)	PE (1)	PE (2)	PS (1)	PS (2)	PI (1)	PI (2)
16:0	33.5	33.7	21.0	21.0	1.5	1.7	40.8	38.9
18:0	13.7	14.0	25.0	25.3	54.0	53.5	10.7	12.6
18:1n-9	31.2	30.7	23.5	23.5	24.9	26.1	7.0	6.1
18:2n-6	16.5	16.3	14.2	14.3	0.7	—	40.9	41.9
20:4n-6	3.4	3.6	11.1	11.5	0.7	0.9	0.7	0.6
22:4n-6	0.2	0.2	0.5	0.5	3.6	3.4	—	—
22:5n-6	0.9	0.9	2.3	2.3	4.1	3.9	—	—
22:5n-3	—	—	0.2	0.1	0.4	0.2	—	—
22:6n-3	0.7	0.7	2.3	1.5	10.1	10.4	—	—

[a]Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.
[b]Nonradioactive PL standards were either directly (1) transmethylated and analyzed by gas chromatography as described in Methods, or first carried twice through the whole procedure (2).
[c]Percent recovery.

N-acyl bonds in SM are stable under the mild conditions used for transmethylation. Furthermore, if a pure preparation of PC is needed, the fraction A can be loaded in 100 μL of chloroform onto a 100-mg silica cartridge preconditioned with hexane. About 80.6% of PC can be eluted with the first 2 mL of acetonitrile/methylacetate/methanolic HCl (130:30:18, by vol), whereas more than 98.6% of SM are retained.

In summary, we further extended a widely used solid-phase extraction method for the separation of the major lipid classes (1,2,7). Making use of differences in polarity, pKa and solvent strength, the separation of PL on the same aminopropyl matrix was refined to the level of individual PL classes. No expensive equipment is needed, and very low solvent volumes suffice. The method considerably simplifies and speeds up the purification of PL in large numbers of sample.

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A Micro Enzymic Method for Determination of Choline-Containing Phospholipids in Serum and High Density Lipoproteins

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A micro method is described for the assay of choline-containing phospholipids in serum and high density lipoproteins (HDL) using an automated microtiter plate reader. The method is adapted from the enzymic method of Takayama, Itoh, Nagasaki, and Tanimuzu (*Clin. Chim. Acta 79*, 93–98, 1977) using phospholipase D, choline oxidase, and peroxidase coupled with the color generating system phenol and 4-amino-antipyrine. The micro method requires 5 μL of serum or HDL sample, and 42 samples can be assayed in duplicate in one run using a 96-well flat-bottom microtiter plate. The reaction is linear up to 400 mg/dL and the lower limit of detection is 0.25 mg of choline-containing phospholipids per assay. The coefficient of variation within an assay is 0.86–0.79%, and day-to-day variation is 0.9–1.5%. Results obtained by the micro method are in excellent agreement with those obtained by the procedure of Takayama *et al.* (r = 0.997). The supernatant left after removal of low density lipoproteins and very low density lipoproteins from serum and precipitation with heparin/manganese chloride reagent can thus be conveniently used for the micro assay of choline phospholipids in HDL.
***Lipids* 28, 949–951 (1993).**

It has been shown that choline-containing phospholipids, including phosphatidylcholine, lysophosphatidylcholine and sphingomyelin, make up 91–97% of serum phospholipids (1,2). For practical purposes, their measurement is therefore a close approximation of total serum phospholipids. To meet the need for routine assays, an enzymic method for measuring choline-containing phospholipids was developed by Takayama *et al.* (1). The method utilizes phospholipase D to release the choline moiety, and the released choline is subsequently oxidized by choline oxidase to form equimolar amounts of betaine and hydrogen peroxide, which in the presence of peroxidase coupled with phenol and 4-aminoantipyrine generates a red color which is amenable to spectrophotometric measurement. This method and its modifications have been used in a number of studies to determine phospholipids in serum and high density lipoproteins (HDL) (2–4).

In the present paper we describe a micro enzymic method adapted from the method of Takayama *et al.* (1) using a microtiter plate reader for the measurement of choline-containing phospholipids in serum and HDL. It uses 5 μL of serum or HDL sample for each determination, and the results are in close agreement with those obtained by the original method (r = 0.997).

In clinical laboratories, a commonly used procedure for measuring HDL cholesterol requires the precipitation of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) from serum with heparin/manganese chloride reagent (H-M reagent) (5). It would be convenient if the same HDL preparation could be used for measurement of phospholipids. However, it has been reported that the H-M reagent interferes with the enzymic assay of cholesterol (6). Since the enzymic assay of choline-containing phospholipids and the assay of cholesterol are based on the same principle, namely the color generated by H_2O_2, we investigated the possible interference of the H-M reagent with phospholipid measurements in HDL by the micro method. Our results show that the interference can be readily overcome, and that it is practical and convenient to use the micro method for measuring choline phospholipids in HDL prepared with the H-M reagent.

MATERIALS AND METHODS

Serum samples. The serum samples used were routine clinical test samples. For developing the micro method, we utilized the supernatant solution that is obtained after precipitation of LDL and VLDL with the H-M reagent for HDL cholesterol measurements. The supernatant was stored in a 4°C refrigerator for at least 24 h after measurement of cholesterol before the fine precipitate was removed by centrifugation at 2000 × *g* for 30 min. The clear supernatant was used for the assay of choline phospholipids in HDL.

Apparatus. The micro enzymic assay utilized a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) linked to an IBM-compatible computer with a 386 microprocessor and a Panasonic KX-P1124 multimode printer. The software, SoftMax, was supplied by the manufacturer of the Microplate Reader. For comparison of the results obtained by the micro method and by the procedure of Takayama *et al.* (1), we tested the same serum samples using the Microplate Reader and a Spectronic 1201 instrument (Milton Roy Co., Rochester, NY).

Chemicals and reagents. All enzymes and chemicals were purchased from Sigma Chemical Company (St. Louis, MO) except that phenol and $CaCl_2 \cdot 2H_2O$ were purchased from Malinckrodt (St. Louis, MO).

A 50 mM Tris/HCl buffer, pH 7.8, solution was prepared that contained 5 g Triton X-100 and 73.51 mg $CaCl_2 \cdot 2H_2O$. A single reagent containing the enzymes and color generating system was prepared fresh each day by dissolving 44 units phospholipase D (from *Streptomyces chromofuscus*), 94 units choline oxidase (from *Arthrobacter globiformis*) and 204 units peroxidase (from horseradish), 5 mg phenol and 14.7 mg 4-aminoantipyrine in 25 mL of the above described buffer.

For the preparation of H-M reagent, 5 g of $MnCl_2 \cdot 4H_2O$ and 1.3×10^5 units of heparin were dissolved in 50 mL of distilled water, and 100 μL of this solution was used for each one mL of serum for precipitation of VLDL and LDL at room temperature for 45 min. The supernatant after centrifugation at 2000 × *g* for 45 min was used for routine measurement of HDL cholesterol.

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Abbreviations: CV, coefficient of variation; HDL, high density lipoproteins; H-M reagent, heparin/manganese chloride reagent; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

The same supernatant was used for developing the micro enzymic method as described under Assay Procedure.

Standard solutions. A standard solution containing 500 mg/dL of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was prepared by dissolving 10.0 mg of the phospholipid in 2 mL of a 5% solution of Triton X-100. Aliquots of this solution were diluted with 5% Triton X-100 solution, to make standard solutions containing 100, 200, 300 and 400 mg/dL of the phospholipid.

Assay procedure. To each well of the microtiter plate (Bio-Rad Laboratories, Richmond, CA) is added with a Hamilton syringe (Baxter Co., McGaw Park, IL) 5 μL of serum, or the HDL supernatant after precipitation of VLDL and LDL, or the standard solution, and then with a Labysystems Multistepper pipet (Baxter Co.) is added 200 μL of the enzyme reagent. All samples, standards and reagent blanks are determined in duplicates. The plate is agitated and incubated at 37°C for 20 min, then read using a 505-nm filter in the Microplate Reader. The results are printed out as directed by the SoftMax program. The average of duplicate measurements is recorded. The duplicates usually differ less than 1%. Duplicates that differ more than 3% are rejected, and the test is repeated.

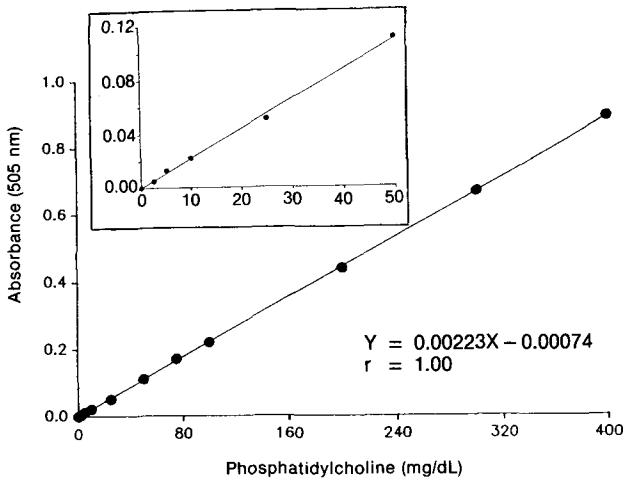

FIG. 2. Standard curve of (A) at 505 nm *vs.* concentration of dipalmitoyl-*sn*-glycero-3-phosphocholine. Reaction time was 20 min at 37°C. The inset is an enlargement of the curve at phosphatidylcholine concentrations below 50 mg/dL.

RESULTS AND DISCUSSION

To select optimal conditions for the micro assay, we varied sample size, the amount of reagent used, incubation time, etc. The assay procedure given above was established on the results of many of such tests.

The time course for the reaction is shown in Figure 1. At a substrate concentration of 400 mg/dL, a plateau of absorbance (A) at 505 nm was reached between 20 to 25 min. At lower substrate concentrations, the plateau was reached within 15 to 20 min. The color was stable for over 1 h, with a slight decline in A after 30 min. For routine assays, we allowed 25-min incubation time before reading.

The results obtained on four standard solutions are shown in Figure 2. Absorbance (A) was linear with substrate concentrations from 2.5 mg/dL up to 400 mg/dL, with the regression line passing through the zero point of both axes ($P < 0.001$). The lower limit of detection was 0.25 mg of choline-containing phospholipids per assay.

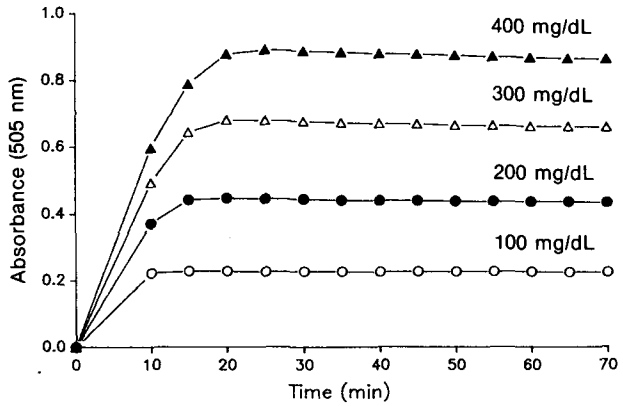

FIG. 1. Time course of the reaction at four concentrations of dipalmitoyl-*sn*-glycero-3-phosphocholine. The phospholipid was dissolved in 5% Triton X-100 and allowed to react with the enzyme reagent at 37°C. Absorbance at 505 nm was read using a Thermomax Microplate Reader.

The coefficient of variation (CV) (standard deviation/mean × 100%) within the assay of 10 determinations for three serum samples was between 0.79 and 0.86%. CV for day-to-day variation for three samples over 12 d was between 0.9 and 1.5% (Table 1).

Figure 3 shows the excellent correlation between the results for 48 serum and samples assayed using Thermomax Microplate Reader the Spectronic 1201 spectrophotometer. The range of values was from 56 to 298 mg/dL; r = 0.997.

TABLE 1

Precision of Measurement of Choline-Containing Phospholipids by the Micro Method[a]

	Within assay variation			Day-to-day variation		
	1	2	3	4	5	6
	101.8	194.3	291.8	102.6	205.3	379.8
	104.1	196.6	299.6	101.2	205.7	387.6
	103.7	198.0	298.3	100.5	213.4	380.7
	104.1	198.4	299.2	102.9	206.3	382.6
	102.7	199.4	299.2	105.3	203.4	377.2
	104.1	198.0	299.6	105.9	206.3	374.6
	103.2	198.4	297.3	104.6	209.3	375.0
	103.7	199.8	300.1	102.5	208.5	376.8
	102.3	200.3	297.8	103.1	206.1	376.7
	102.7	198.0	300.5	104.2	209.5	377.7
				104.7	210.4	381.6
				104.6	207.3	379.5
Mean	103.3	198.1	298.4	103.5	207.6	379.2
SD	0.8	1.7	2.5	1.6	2.6	3.5
CV (%)	0.79	0.86	0.84	1.5	1.3	0.9

[a]All values are in mg/dL and are the average of duplicate measurements. Serum samples 1-3 were each measured 10 times successively on the same day, and serum samples 4-6 were measured each day for 12 d; CV, coefficient of variation.

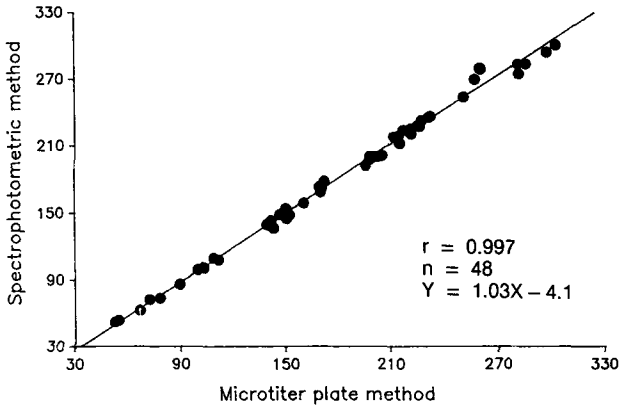

FIG. 3. Correlation between results obtained by the microtiter plate method and the spectrophotometric method. A Thermax Microplate Reader and a Spectronic 1201 spectrophotometer were used.

Two problems were encountered in the determination of choline-containing phospholipids in HDL prepared using the H-M reagent. One was the fine precipitate that continued to form after removal of the precipitates of VLDL and LDL, which caused turbidity and interfered with the readings of the Microplate Reader and led to erratically high results. We solved this problem by storing the HDL preparation in the refrigerator at 4°C for at least 24 h to allow complete precipitation before removal of the fine precipitate by centrifugation at 2000 × *g* for 30 min. The clear supernatant was then used for the measurement of choline phospholipids. Determination of phospholipids by the chemical method based on measuring inorganic phosphorus (7) showed that removal of the fine precipitates did not change the phospholipid content of the HDL preparation (Table 2). It has been reported previously that after removal of apo B-containing lipoproteins from serum by precipitation with the H-M reagent, the supernatant continues to become turbid due to formation of manganese oxide (5). The turbidity eventually turns into the fine precipitate described here.

The other problem in measuring HDL phospholipids was the interference of the H-M reagent with the color generated by H_2O_2 which is the end product of the enzymic reactions for both the measurement of cholesterol and of choline-containing phospholipids. Previously, McGowan *et al.* (6) had shown that the H-M reagent caused the values of HDL cholesterol measured by the enzymic method to be somewhat high. In our experiments, we also found the H-M reagent to cause slightly higher absorbance readings, which were equivalent to less than 3% of the phospholipids. This difference was corrected by adding 7 μL of H-M reagent to 3 mL of the enzyme mixture to simulate the concentration of H-M reagent in the HDL supernatant. This modified enzyme mixture was used for the standard assay, so that the effect of the H-M reagent could be canceled automatically in the calculations using the SoftMax computer program.

The micro method described here is simple and expedient, and is suited for the routine measurement of choline-containing phospholipids in serum and HDL. It can easily be adapted to measure other lipoprotein fractions and body fluids as well. The method offers a number of advantages over the original enzymic method of Takayama *et al.* (1). The sample size required for each determination is reduced from 20 to 5 μL, and the amount of enzyme reagent required is about one-fourth. An important advantage is also the expedience gained by use of the microtiter plate reader. In seconds, 42 serum samples in duplicates besides standards and blanks can be read in a single 96-well microtiter plate. Interfacing of the instrument with a computer and printer eliminates much of the tedium of routine operations.

Another micro method for the quantitation of phospholipids that uses a microtiter plate fluorometer has recently been reported (8). This method is highly sensitive and requires dilution of the serum before the measurements. The micro method described here is less sensitive and uses serum directly without dilution.

Phospholipids, especially choline phospholipids, are major components of lipoproteins, and of cellular membranes and organelles. Although the relevance of serum choline phospholipid levels to disease is largely unknown, it deserves further investigation. The micro enzymic method described here may expedite such investigations.

TABLE 2

Concentrations of HDL Phospholipids Before and After Removal of the "Fine Precipitate"[a]

Sample	Before	After	Difference
1	94.7	93.5	1.2
2	113.7	113.1	0.6
3	102.0	102.8	−0.8
4	125.0	123.8	1.2
5	119.5	120.9	−1.4
6	69.7	69.5	0.2
7	108.7	109.4	−0.7
8	83.0	83.0	0.0
9	104.6	108.3	−3.7
10	91.6	92.1	−0.5
11	138.4	139.7	−1.3
12	97.9	96.5	1.4
13	100.5	100.5	0.0
14	176.9	178.4	−1.5
15	127.6	126.4	1.2
		Mean	−0.27
		SD	1.37

[a]Values for phospholipids are in mg/dL and were calculated based on inorganic phosphorus as determined according to the method of Dittmer and Wells (7); HDL, high density lipoprotein.

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Microwave-Mediated Methanolysis of Lipids and Activation of Thin-Layer Chromatographic Plates

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A technique is described for the methanolysis of fatty acids from acylglycerols with HCl/CH_3OH or $NaOH/CH_3OH$ using a microwave oven. The esterification is rapid and complete and does not result in significant degradation of polyunsaturated fatty acids, even in the presence of oxygen. The fatty acid compositions of intact tissues were also determined using this technique. The microwave oven has also been used to condition normal silica gel and argentation thin-layer chromatographic plates in a fraction of the time normally required.
Lipids 28, 953–955 (1993).

In our work on the effect of environmental and genetic factors on glycerolipid metabolism in higher plants, we routinely analyze large numbers of samples of lipids and their fatty acids. Existing methods of lipid analysis are often time-consuming, especially if thin-layer chromatographic (TLC) plates are prepared in the laboratory. Results obtained are also often inconsistent and unreliable because of the oxidation of unsaturated fatty acid that can occur. Activation of TLC plates by standard techniques may take 1–4 h (1,2) and the preparation of fatty acid methyl esters (FAME) from lipids 1–8 h at 80°C in H_2SO_4/CH_3OH or HCl/CH_3OH (1,3,4).

The microwave oven has been used in the past in the laboratory to speed up synthetic reactions in the preparation of various organic compounds (5). Recently, it has also been used to reduce the reaction time for the hydrolysis of triacylglycerols, the esterification of free fatty acids and for other chemical transformations of long-chain fatty acid esters (6). We have investigated the routine use of microwaves to speed up the esterification of fatty acids from plant tissue. The technique described here ensures complete esterification of acylglycerols in a short period of time without oxidation using lower concentrations of reagents.

We have also found that microwaves can be used to reduce the time required for the activation of TLC plates as compared to using a conventional oven.

MATERIALS AND METHODS

Lipid separation by TLC. Lipids were extracted from leaf tissues, seeds and cyanobacterial cell cultures with chloroform/methanol (2:1, vol/vol), and the nonlipid contaminants were removed from the total lipid extract as described previously (7). Silica gel G (E. Merck, Darmstadt, Germany) plates were prepared in the laboratory with ammonium sulfate (1.5 M) (2) using an applicator from Desaga (Heidelberg, Germany). The plates were activated for 10 min in a microwave oven (Toshiba, Toronto, Canada, Model ERX-8900) at full power or, for comparison, for 4 h in a conventional oven at 110°C. Plates were cooled to room temperature and used immediately or stored for later use.

For molecular species analysis of the lipids separated, silica gel plates were impregnated with 10% $AgNO_3$ (w/w) and activated in the microwave oven for 10 min. The chromatoplate was developed with chloroform/methanol/water (65:25:4, by vol), the silica gel bands containing the lipid fractions were then scraped from the plate directly into glass tubes and the samples were methanolyzed with 0.5 mL $NaOH/CH_3OH$ (0.1 M).

Methanolysis of lipids and methylation of fatty acids. Aliquots of lipid or fatty acid samples were methanolyzed or methylated in thick glass tubes (20 × 150 mm with Teflon-lined screw caps; Pyrex, Corning, NY) with freshly prepared HCl/CH_3OH or $NaOH/CH_3OH$. The samples were exposed to the maximum power of the microwave oven for 1 min. For safety, the glass tubes containing lipid samples were encased in a large microwave-safe plastic container. The volume of HCl/CH_3OH *must not* exceed 0.5 mL because excess reagent and rapid heating may lead to the explosion of the tubes. After methanolysis, the samples are cooled to room temperature inside the microwave oven; the hot tubes should *not* be removed from the microwave oven immediately after heating. We have experienced no explosions of tubes using these simple precautions. Identical samples were methanolyzed in a conventional oven at 80°C for 6–8 h comparison. Whole *Brassica napus* seeds were directly methanolyzed after being crushed with a smooth glass rod in the glass tube with HCl/CH_3OH. The resulting FAME were extracted with hexane and quantified by gas–liquid chromatography (GLC) using a fused silica capillary column (DB 225, 30 m × 0.25 mm; J & W Scientific, Folsom, CA) programmed with an initial hold for 2 min at 180°C, and then temperature was increased to 220°C at the rate of 4°C/min (Hewlett-Packard GLC, Model 5890A; Mississauga, Ontario, Canada). Pentadecanoate (15:0) was used as internal standard.

Chemicals. All solvents were obtained from Fisher Scientific Co. (Unionville, Ontario, Canada) and were glass distilled before use. A model mixture of free fatty acids (Sigma Chemical Co., St. Louis, MO) ranging from C_{12} to C_{20} was prepared in the laboratory, HCl/CH_3OH was prepared in the laboratory by passing dry HCl gas through dry, distilled CH_3OH.

RESULTS AND DISCUSSION

TLC plate activation. Our objective was to minimize the time required for TLC plate activation without reducing the quality of lipid separation. Activation of 0.5-mm thick silica gel TLC plates for 10 min at full power in the microwave oven resulted in a quality of separation of individual lipids equal to that occurring with at least 4 h at 110°C in a conventional oven (2). TLC plates impregnated with 10% $AgNO_3$ were also activated for 10 min in the microwave oven; the separation of lipid molecular species on these plates was identical to that obtained by the

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Abbreviations: FAME, fatty acid methyl ester(s); GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

TABLE 1

Total Fatty Acid Methyl Esters Recovered from Different Quantities of Free Fatty Acids Using Conventional and Microwave Ovens[a]

Fatty acid added	Determined	
	Conventional	Microwave
0.5	0.5 ± 0.1[b]	0.5 ± 0.1
1.0	1.0 ± 0.1	0.9 ± 0.0
1.5	1.5 ± 0.0	1.5 ± 0.1
2.0	2.0 ± 0.1	2.0 ± 0.1
2.5	2.5 ± 0.1	2.5 ± 0.1
3.0	2.9 ± 0.1	3.1 ± 0.1
3.5	3.4 ± 0.1	3.6 ± 0.2
4.0	3.7 ± 0.1	4.0 ± 0.2
4.5	4.2 ± 0.2	4.4 ± 0.3
5.0	4.7 ± 0.1	4.8 ± 0.3

[a]Different volumes of a fatty acid mixture were analyzed by gas-liquid chromatography (GLC) after methanolysis in HCl/CH_3OH as described in Materials and Methods; the added quantities are compared to the values determined by GLC. The complete fatty acid analyses are contained in Table 2.
[b]Mean ± SD, n = 5.

conventional method and the rapid procedure resulted in less discoloration of the chromatoplates.

Preparation of FAME. Different concentrations of a mixture of fatty acids, ranging in carbon chain length from C_{12} to C_{20}, were methylated with HCl/CH_3OH (1.5 M) for 1 min in the microwave oven and 6-8 h in a conventional oven (Table 1). Higher concentrations of fatty acids were shown to require up to 2 min for maximum yield (data not shown). There was no significant change in the quantity of the fatty acids between mixtures methylated in a microwave and in a conventional oven although the mean values of the microwave method were closer to the added values at higher concentrations.

The yield of FAME by the microwave method was examined at various concentrations of HCl/CH_3OH in order to determine the optimal concentration of the reagent (Table 2). The quantities of FAME produced at different concentrations of HCl/CH_3OH reagent, ranging from 0.025 to 2.0 M, were quite similar. Higher concentrations of HCl/CH_3OH appeared to cause some loss of FAME (data not shown) while the low yield of FAME at concentrations of HCl/CH_3OH at 0.00625 M was probably due to incomplete methylation. Our results suggest that 0.5 M HCl/CH_3OH is a suitable concentration which yields optimum levels of FAME from lipids without loss of unsaturated fatty acids.

Analysis of whole plant tissue. The analyses of the fatty acid content of *B. napus* seeds did not require prior lipid extraction and purification. The seeds were methanolyzed directly with 1.5 M HCl/CH_3OH or 0.1 M $NaOH/CH_3OH$ in the microwave oven. The results of GLC analyses (Table 3) showed no significant differences in the fatty acid composition of seed lipids methanolyzed by these reagents. There was a small difference in quantity, probably due to the inability of $NaOH/CH_3OH$ to methylate free fatty acids (unpublished data).

Loss of unsaturated fatty acids by oxidation. The model fatty acid mixture was esterified using the microwave method and in a conventional oven in tubes that were incompletely closed (unsealed). In unsealed tubes from the microwave oven, the analyses revealed no significant oxidation of unsaturated fatty acids, while methylation of fatty acids by the conventional method in unsealed tubes caused severe loss of unsaturated fatty acids due to oxidation (data not shown). In the microwave method, the duration of heating is only 1 min and therefore, there is less chance of evaporation of the regent and subsequent oxidation of unsaturated fatty acids should this occur.

Methanolysis of molecular species in the presence of $AgNO_3$. The molecular species of galactosyldiacylglycerols and phospholipids are routinely analyzed in our laboratory using sodium methoxide (8,9) (which does not react with $AgNO_3$). This technique is time-consuming and sometimes results in the appearance of false peaks due to impurities in the prepared sodium methoxide reagent. The use of $NaOH/CH_3OH$ in the analysis of lipid molecular species using the microwave oven was faster,

TABLE 2

Recovery of Fatty Acid Methyl Esters Prepared from a Mixture of Free Fatty Acids Using Various Concentrations of HCl/CH_3OH[a]

Fatty acid	Concentration of HCl/CH_3OH					
	2.0M	1.5M	0.5M	0.1M	0.025M	0.00625M
	(mol%)					
12:0	3.8 ± 0.3[b]	3.8 ± 0.1	3.8 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1
14:0	19.6 ± 0.3	19.4 ± 0.2	19.5 ± 0.2	20.4 ± 0.1	20.0 ± 0.4	19.9 ± 0.3
16:0	6.0 ± 0.3	5.9 ± 0.1	5.9 ± 0.0	6.0 ± 0.0	5.9 ± 0.1	6.0 ± 0.1
16:1*c*[c]	7.3 ± 0.1	7.3 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	7.5 ± 0.1	7.4 ± 0.1
16:1*t*[c]	10.2 ± 0.1	10.4 ± 0.1	10.5 ± 0.1	10.8 ± 0.1	10.7 ± 0.1	10.7 ± 0.1
17:0	6.8 ± 0.1	6.7 ± 0.1	6.7 ± 0.0	6.6 ± 0.0	6.6 ± 0.1	6.7 ± 0.1
18:0	1.5 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.1	1.4 ± 0.0	1.4 ± 0.0
18:1	17.6 ± 0.2	17.7 ± 0.1	17.6 ± 0.1	17.3 ± 0.1	17.4 ± 0.2	17.4 ± 0.2
18:2	13.6 ± 0.1	13.8 ± 0.1	13.9 ± 0.1	13.4 ± 0.1	13.7 ± 0.1	13.6 ± 0.1
18:3	8.5 ± 0.1	8.8 ± 0.1	8.9 ± 0.0	8.5 ± 0.1	8.6 ± 0.1	8.6 ± 0.1
20:0	4.9 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	4.1 ± 0.1	4.2 ± 0.2	4.2 ± 0.1
Total (μmole)	1.3 ± 0.05	1.3 ± 0.1	1.4 ± 0.03	1.3 ± 0.0	1.3 ± 0.0	0.7 ± 0.1

[a]All samples were methanolyzed in 0.5 mL of reagent for 1 min in a microwave oven at full power.
[b]Mean ± SD, n = 5.
[c]16:1*c*, *cis*-9-hexadecenoic acid; 16:1*t*, *trans*-9-hexadecenoic acid.

TABLE 3

FAME Prepared from Intact *Brassica napus* Seeds[a]

Fatty acid	Methanolysis reagent	
	1.5M HCl	0.1M NaOH
	(mol%)	
16:0	5.3 ± 0.2[b]	5.3 ± 0.2
16:1*c*[c]	0.3 ± 0.0	0.2 ± 0.1
16:1*t*[c]	0.0 ± 0.0	0.0 ± 0.1
16:2	0.1 ± 0.0	0.1 ± 0.0
16:3	0.1 ± 0.0	0.1 ± 0.0
18:0	1.6 ± 0.0	1.6 ± 0.0
18:1	62.5 ± 0.2	62.4 ± 0.3
18:2	20.5 ± 0.0	20.4 ± 0.2
18:3	8.1 ± 0.0	8.1 ± 0.0
20:0	0.5 ± 0.1	0.5 ± 0.1
20:1	1.0 ± 0.0	1.0 ± 0.1
Total (μmoles)	30.3 ± 1.7	28.3 ± 1.4

[a]The fatty acid methyl esters (FAME) were prepared using 1.5M HCl/CH_3OH and 0.1M $NaOH/CH_3OH$ in the microwave oven.
[b]Mean ± SD, n = 5.
[c]As in Table 2.

more reliable and the GLC analyses of FAME were free from contamination (data not shown).

In conclusion, the microwave method of lipid analysis has proven to be very efficient, fast and reliable. The technique requires very little reagent to esterify lipids and free fatty acids in a short period of time. We have also successfully employed the method to analyze fatty acid contents of intact seeds, whole leaves and cyanobacterial cells in fresh sedimented pellets from liquid cultures.

ACKNOWLEDGMENT

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LETTER TO THE EDITOR

Comments on Erythrocyte Membrane Phospholipid Fatty Acid Changes in Cerebral Palsy Patients During Nutritional Rehabilitation

Dear Sir:

When Harper *et al.* (1) reported on the fatty acid profile of erythrocyte membrane phospholipids in cerebral palsy, they showed that although a soy oil-based nasogastric formula resulted in an increased proportion of linoleic acid in the erythrocyte phospholipids, α-linolenic acid and 20 and 22 carbon polyunsaturated fatty acids were unchanged over a period of at least 25 d on this regimen. It was suggested that the high linoleic acid in the formula may have inhibited the desaturases, causing the observed lack of change in the longer chain metabolites.

Two other factors may also have contributed to the increase in linoleic acid, but lack of change in α-linolenic acid and in 20 and 22 carbon ω6 and ω3 fatty acids. First, as these children were gaining weight and body fat during this phase of nutritional rehabilitation, they were not in a steady-state. In humans in the steady-state, dietary linoleic acid is more readily oxidized than are saturated fatty acids (2). Although not studied in humans, α-linolenic acid is also more readily oxidized than are saturated acids (3). A greater demand in these patients to oxidize dietary linoleic and α-linolenic acids, as provided in the soy oil-based formula, might contribute to a lack of change in both α-linolenic acid and longer chain metabolites of both linoleic and α-linolenic acid during nutritional rehabilitation in cerebral palsy.

Second, various vitamins and trace elements are required in linoleic acid desaturation. Notable among these is zinc, the deficiency of which has a clear inhibitory effect on the synthesis of both arachidonic and docosahexaenoic acids in animals and in humans (4,5). Although the cerebral palsy patients reported by Harper *et al.* (1) may not have been zinc deficient, their weight gain during the period of the study again points to a nonsteady-state for zinc which could influence the availability of zinc needed in desaturation. This could also contribute to the lack of change in longer chain metabolites of both linoleic and α-linolenic acid in the presence of adequate amounts of these precursors in the diet.

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Erythrocyte Membrane Phospholipid Fatty Acid Changes in Cerebral Palsy Patients During Nutritional Rehabilitation—A Response

Dear Sir:

We are very interested in the suggestions of Dr. Cunnane (1). The whole issue of nutrient supply in relation to growth rate is very important and often misunderstood, so we welcome the opportunity to clarify this point.

Although our patients were all very deficient in body mass before treatment, unlike children in the developing world, there was no clinical evidence of deficiency of minor components; in particular, hair, skin and mucous membranes were all healthy.

Our treatment regime did not lead to extremely rapid weight gain (30 g/kg/d, or more) but was usually around 10 g/kg/d. At that rate, the enteral feed used, Isocal™ (Mead Johnson, Evansville, IN), provides sufficient trace and minor nutrients to synthesize lean tissue. On average, the children studied were consuming 15.7 mg Zn per day. Isocal™ provides 10 mg Zn per 1000 kcal; the mixed Canadian diet contains approximately 5 mg Zn per 1000 kcal (2), and in a study involving Canadian preschool children, aged 4–5 y, the average Zn intake was 4.6 ± 1.0 mg per 1000 kcal (3). In addition, we do not base our prescription of nutrient intake on recommended intakes (which are designed to supply average, normal requirements plus two SD), but on calculations based upon data describing tissue composition and percent absorption from the gut. We are therefore confident that there were no minor nutrient deficiences.

Isocal™ provides 37.4% of energy as fat. Of the fatty acids therein, 18:2n-6 and 18:3n-3 comprise 45.6 and 6.7%, respectively (4). There does not seem much likelihood that there was a limitation in the supply of these fatty acids.

We thank Dr. Cunnane for his comments and hope that our response makes it clear that the provision of micronutrients is important and needs careful control in refeeding protocols.

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CORRECTIONS

Effect of Dietary α-Linolenic Acid on n-3 Fatty Acids of Rainbow Trout Lipids
Karen C. Sowizral, Gary L. Rumsey and John E. Kinsella
Lipids 25, 246-253 (1990)

In the Results and Discussion section of this paper, part of the third paragraph on page 249 and part of the first paragraph on page 250 were inadvertently deleted. The third and fourth paragraphs of the Results and Discussion section should read:

The mean weights of the fish on the four diets were similar, and all fish showed comparable weight gains (76 g) during the study period. Feed conversion (weight food fed/weight gain) for fish on the four diets was not statistically significant between treatments and averaged 1.30. There was no significant effect of diet on the proximate composition (Table 4).

There were no significant differences in the lipid content of muscle from fish fed different diets though lipid content of the flesh increased from 4.3 ± 0.14% to 6.95 ± 0.21% over the 64-day feeding period. The lipid content was within the range reported by this and other laboratories (22,23).

Acute Toxicity of *trans*-5-Hydroxy-2-nonenal in Fisher 344 Rats
Akiyoshi Nishikawa, Rama Sodum and Fung-Lung Chung
Lipids 27, 54-58 (1992)

The title of the paper should read:

Acute Toxicity of *trans*-4-Hydroxy-2-nonenal in Fisher 344 Rats

The Esterified Plasma Fatty Acid Profile Is Altered in Early HIV-1 Infection
Michael D. Peck, Emilio Mantero-Atienza, Maria Jose Miguez-Burbano, Mary Ann Fletcher, Gail Shor-Posner and Marianna K. Baum
Lipids 28, 593-597 (1993)

In this paper, one of the authors' names was inadvertently omitted. The correct sequence of authors and their affiliations is:

Michael D. Peck[a], Emilio Mantero-Atienza[b,c], Maria Jose Miguez-Burbano[b], Ying Lu[b,c], Mary Ann Fletcher[c], Gail Shor-Posner[b,c] and Marianna K. Baum[b,c]
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A Multivariate Optimization of Triacylglycerol Analysis by High-Performance Liquid Chromatography
Magnus H.J. Bergqvist and Peter Kaufmann
Lipids 28, 667-675 (1993)

On page 669, Equation 2 should read:

$$\beta = (X^TX)^{-1}X^TY$$

On page 673, in Figure 6A, peaks numbers 15 and 16 should be numbers 16 and 17, respectively.

ARTICLE

Sterol Synthesis and Viability of *erg11* (cytochrome P450 lanosterol demethylase) Mutations in *Saccharomyces cerevisiae* and *Candida albicans*

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The identification of the precise structural features of yeast sterol molecules required for the essential "sparking" function has been a controversial area of research. Recent cloning and gene disruption studies in *Saccharomyces cerevisiae* have shown that C-24 methylation (*ERG6*), C-5 desaturation (*ERG3*) and Δ^8-Δ^7 isomerization (*ERG2*) are not required, while C-14 demethylation (*ERG11*) and C-14 reduction (*ERG24*) are each required for aerobic viability. Earlier observations had indicated that C-14 demethylase deficient strains could be restored to aerobic growth by suppressor mutations that caused a deficiency in C-5 desaturase. These strains were reported to synthesize some ergosterol, indicating that they contained leaky mutations in both *ERG11* and *ERG3*, thereby making it impossible to determine whether the removal of the C-14 methyl group was required for aerobic viability. The availability of the *ERG11* and *ERG3* genes has been used in this study to construct strains that contain null mutants in both *ERG11* and *ERG3*. Results show that these double disruption strains are viable and that spontaneously arising suppressors of the *ERG11* disruption are *erg3* mutants. The *erg11* mutants of *S. cerevisiae* are compared to similar mutants of *Candida albicans* that are viable in the absence of the *erg3* lesion.
***Lipids* 28, 963-967 (1993).**

The ergosterol biosynthetic pathway of yeast has been a focus of study for several decades. Initial interest resulted from the fact that several antifungal agents target this pathway or its end product, ergosterol. This is particularly true of the portion of the pathway beginning with the formation of the first sterol molecule, lanosterol. Although research on the mechanisms of action and resistance for antifungals and the development of new agents is still pursued, additional fundamental questions related to sterol function in membranes are being addressed.

Sterols play critical bulk roles in eucaryotic membranes providing the appropriate membrane fluidity to allow for normal functions such as membrane permeability (1,2), the activity of membrane-bound enzymes (3) and cellular growth rates (4). Beyond the bulk function, sterol has been implicated in providing an essential component required for completion of the cell cycle. Studies using a sterol auxotroph have indicated that yeast cells were unable to exit the G1 phase of the cell cycle unless a specific sterol molecule was present (5). This specific sterol was required in hormonal amounts and allowed resumption of the cell cycle provided that sufficient nonspecific bulk sterol was also present. Ergosterol could satisfy both the bulk function and the specific, or "sparking" function (6,7).

Several sterol structural features have been suggested as essential for sparking activity. One group proposed that methylation of the C-24 position (*ERG6* gene) was essential (8,9) while a second proposed that desaturation of the C-5(6) position (*ERG3* gene) was required for sparking (6,7,10). Recently the *ERG6* and *ERG3* genes have been cloned and used to obtain gene-disrupted haploid strains (11,12). In both cases, cells carrying the null allele were viable, indicating the nonessentiality of each gene. A similar result has also been reported for gene *ERG2* encoding the Δ^8-Δ^7 isomerase activity (13). More recent reports (14,15) describe the cloning and disruption of the C-14 sterol reductase gene (*ERG24*). In one of these studies (14), cells containing the C-14 reductase gene disruption were shown to be nonviable under aerobic conditions, indicating that this gene contributes an essential structural feature to the sparking function.

Moreover, the C-14 demethylase (*ERG11*), has been cloned and sequenced (16). Haploid strains disrupted for this gene are nonviable under aerobic conditions (16). Although this result established an essential role for this gene, prior studies using several aerobically viable *erg11* mutant strains (17,18) indicated that these mutants also carried a mutation in *ERG3*, the gene for C-5(6) desaturase. An explanation for this situation has been proposed based on the isolation and identification of a novel sterol, 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol (19), found in *erg11* mutants of *Candida albicans* (20,21). Although not a problem for *C. albicans*, production of this sterol in *erg11* mutants of *Saccharomyces cerevisiae* is suggested to result in loss of viability due to the sterol's interference with normal membrane function. Its formation in *erg11* strains presumably arises from the action of C-5(6) desaturase on C-14 methyl fecosterol rather than its normal substrate, fecosterol, with the resulting formation of the diol. Indeed, this diol has been found in a wild type *Saccharomyces* strain treated with the 14α-demethylase inhibitor, fluconazole (19). The presence of an *erg3* mutation that prevents the formation of the diol in *S. cerevisiae erg11* mutants thus allows viability of the cell.

One of the features of the double mutants used in prior studies (17,18) is that they were reported to continue to produce some ergosterol, indicating that both the *erg11* and *erg3* mutations were leaky. This presents a difficulty in interpretation since sparking sterol is required in such small amounts and also presents a problem in determining the level of diol required to render the cell inviable. To clarify such questions we have (i) constructed *S. cerevisiae*

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Abbreviations: BSTFA, *N,O-bis*(trimethylsilyl)trifluoroacetamide; GC, gas chromatography; MS, mass spectroscopy; PCR, polymerase chain reaction, *sld*, suppressor of lanosterol demethylation; TMCS, trimethylchlorosilane; Tween 80, polyoxyethylenesorbitan monooleate; YMG, galactose minimal medium; YMM, yeast minimal medium; YPD, yeast extract, peptone, dextrose.

erg11 erg3 double disruptions (nonleaky null mutants) and various combinations of the disruptions and point mutations, (ii) isolated and investigated the nature of suppressors of the *erg11* disruption and (iii) re-examined the sterol profiles of a *C. albicans erg11* mutant. Our results confirm the aerobic viability of *erg11 erg3* null mutant strains and present a new array of accumulated sterol intermediates which is compatible with growth.

MATERIALS AND METHODS

Strains. The strains used in this study and the sterol biosynthetic lesions they contain are listed in Table 1. Strain *erg3Δ::LEU2 erg11::URA3* was created by disruption of the *ERG11* gene site in strain *erg3Δ::LEU2* (see below). Strain *erg3 erg11::URA3* was created by crossing *erg3-2c* (12) and the *erg11:URA3* strain. Spontaneous revertants of *erg11::URA3* to aerobic viability were isolated by collecting anaerobically-grown cells and plating them on yeast minimal medium (YMM) under aerobic conditions. After 5–7 d colonies emerged from the nongrowing lawn. The variants were confirmed to retain the *erg11::URA3* genotype and were designated *s*uppressors of *l*anosterol *d*emethylase *d*eficiency (*sld*) strains.

Growth. Strains of *S. cerevisiae* were grown aerobically on 1% yeast extract, 2% peptone and 2% dextrose (YPD) or 0.67% yeast nitrogen base and 2% dextrose (YMM) or 2% galactose (YMG) at 30°C. For anaerobic growth, YMM medium was boiled, degassed and then supplemented with ergosterol and Tween 80 added from a 2% ergosterol solution in ethanol/Tween 80 (1:1, vol/vol) to a final ergosterol concentration of 35 mg/L, giving a final Tween 80 concentration of 0.0875% (22). Media were supplemented with the appropriate nutritional supplements when selection protocols were employed. Strain D10 of *C. albicans* (23) was maintained on YPD supplemented with 45 mg/mL nystatin to prevent reversion of the *erg11* lesion.

Gene disruption. Disruption of the *ERG11* gene in *erg3Δ::LEU2* was accomplished as previously described (16) using a modification of a previously described electroporation procedure for the transformation step (24). Briefly, a 1.1 kb fragment of the *URA3* gene from YEp24 was inserted into the coding sequence of *ERG11* (LB4-3A). A linear *Bam*HI-*Hind*III fragment from this construct was used to transform yeasts to uracil prototrophy. The *ERG3* gene was also disrupted as previously described (12). A 1.1 kb *SAl*I-*Xho*I fragment spanning the *ERG3* gene was deleted, the *Xho*I site was filled in and in its place was inserted a 2.0 kb *Sal*I-*Hpa*I fragment containing the *LEU2* gene. Cells were grown aerobically to late log phase at 30°C, pelleted and suspended in 0.3 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 10% glycerol for transformation (25). Selection of the uracil prototrophs was done aerobically. Gene disruption was confirmed using polymerase chain reaction (PCR; results not shown).

PCR. PCR procedures were carried out in a Perkin Elmer (Norwalk, CT) Cetus DNA Thermal Cycler. Amplimers based upon the published *ERG3* sequence (12) were chosen to yield a 2.2 kb amplification product. The *Kpn*I and *Sph*I restriction enzyme sites were included in the amplimers to facilitate proper orientation upon cloning into an expression vector. These primers were used to amplify *sld1*-1 genomic DNA.

Analysis of amplified alleles. Following amplification of the wild-type *ERG3* and *sld1*-1 from the respective strains, the products were inserted into a galactose inducible plasmid, pYES2.0 (Invitrogen). Ten of 12 *ERG3* and nine of 12 *sld1-1 Escherichia coli* transformants were shown to contain PCR products in the correct orientation. From these, six of each were transformed into a *erg3Δ::LEU2 ura3* Gal^+ host. The presence of a functional *ERG3* allele was established based upon the restoration of cycloheximide resistance. Minimum inhibitory concentration values of the transformants were obtained using YMM or YMG as growth media.

Sterol analysis. Cells for sterol analysis were grown to stationary phase in YPD medium. After harvesting by centrifugation, the cells were subjected to saponification as previously described (26). Gas chromatographic (GC) separation and mass spectral (MS) analysis of sterols were done on a Finnigan MAT TSQ 700 (San Jose, CA) GC/MS. GC separations were carried out on a 15 m × 0.32 mm Durabond DB-5 column with a 0.25-mm thickness. The oven temperature was programmed from 50–260°C at 25°C per min and held at 260°C for 20 min. The carrier gas was helium at a linear velocity of 60 cm/s. The capillary column was interfaced directly into the mass spectrometer ion source. The indicated ion source temperature was 150°C. The MS was operated in the electron impact mode at 70 eV and was scanned from *m/z* 50 to *m/z* 650 at a 0.5 s/scan rate. The resulting spectra were enhanced by subtraction when necessary. The chromatographic peaks were quantitated based on area percent. Derivatizations were done with *N, O-bis*(trimethylsilyl)-

TABLE 1

Strains of *Saccharomyces cerevisiae* Used in this Study[a]

Strain	Ergosterol mutation	Source
erg3-2c	*erg3* Mutation	Arthington *et al.* (12)
erg3Δ::LEU2 (LB4-3A)	*erg3* Disruption	Arthington *et al.* (12)
JR4	*erg3 erg11* Mutations	Taylor *et al.* (18)
SG1	*erg3 erg11* Mutations	Trocha *et al.* (17)
erg3-2c erg11::URA3	*erg3* Mutation *erg11* disruption	This study
erg3Δ::LEU2 erg11::URA3	*erg3 erg11* Disruptions	This study
erg11::URA3	*erg11* Disruption	Kalb *et al.* (16)
erg11::URA3 sld1	*erg11* Disruption *sld1*	Turi (27)
sld1-1	*erg3* Mutation	Strain TTY202A of Turi (27)
CG379	Wild-type parent for *erg3* strains	C. Giroux (Yeast Genetic Stock Center)

[a]Abbreviation: *sld*, suppressor of lanosterol demethylation.

trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) purchased from Pierce (Rockford, IL). Fifty mL of BSTFA containing 1% TMCS was added to the dry sample followed by 50 mL of pyridine. Solutions were heated for 5 min at 45°C on a heating block. The sample was cooled to room temperature before analysis.

RESULTS AND DISCUSSION

Previously isolated lanosterol demethylase mutants of *S. cerevisiae* (17,18) have been reported to be leaky and to contain a second leaky mutation in the C-5(6) desaturase (*erg3*). The resulting formation of low levels of ergosterol makes conclusions regarding the essential sterol structure provided by the *ERG11* and *ERG3* genes problematic. Single disruptions of these genes result in aerobic viability for *erg3* (12) and aerobic nonviability for *erg11* (16). The combination of these two disruptions would clarify the proposed *erg3* suppression of *erg11* mutants and could help test the hypothesis that the *erg11* mutation results in nonviability due to the accumulation of a sterol diol (19). To that end, several combinations of *ERG11* disruptions and *ERG3* disruptions and point mutations as well as a suppressor of the *erg11::URA3* aerobic lethality were generated and characterized as to sterol content and aerobic viability. In addition, a lanosterol demethylase mutant of *C. albicans* was analyzed to ascertain how closely sterol metabolism and requirements are related in the two species.

Table 2 shows the sterol profiles of the strains used in this study. The *erg11::URA3* strain is not included since it is not viable under the aerobic conditions required for sterol synthesis. Strains bearing the *erg3* mutation or the disrupted *ERG3* gene copy produce similar typical sterol profiles. Strain JR4 accumulated exclusively 14-methylsterols indicative of a block in lanosterol demethylation. Trace amounts of ergosterol were also detected confirming that the mutation is leaky (18). No evidence of the presence of a sterol diol was observed. The *erg3 erg11* strains produced for this study, one containing the *erg3* point mutation and the *ERG11* disruption (*erg3-2c erg11::URA3*) and one containing disruptions of both genes (*erg3Δ::LEU2 erg11::URA3*), produced the same

TABLE 2

Sterols Accumulated[a] by Ergosterol Mutants of *Saccharomyces cerevisiae*

Strain	Accumulated sterols
erg3-2c	65% Ergosta-7,22-dien-3β-ol 15% Ergosta-8,22-dien-3β-ol 10% Ergosta-8-en3β-ol 6% Ergosta-8,24(28)-dien-3β-ol 4% Ergosta-7-en-3β-ol
erg3Δ::LEU2	74% Ergosta-7,22-dien-3β-ol 7% Ergosta-8,24(28)-dien-3β-ol 6% Ergosta-8,22-dien-3β-ol 4% Ergosta-7,24(28)-dien-3β-ol 4% Ergosta-7,22,24(28)-trien-3β-ol 4% Ergosta-8,22,24(28)-trien-3β-ol
JR4	64% 14-Methylfecosterol 12% Obtusifoliol 24% Lanosterol Ergosterol (trace)
SG1	42% Ergosta-7,22-dien-3β-ol 31% Ergosta-7-en-3β-ol 18% Ergosta-8-en-3β-ol 5% Ergosta-8,24(28)-dien-3β-ol 2% Lanosterol 1% Ergosta-8,22-dien-3β-ol/zymosterol <1% Ergosterol
erg3-2c erg11::URA3	67% 14-Methylfecosterol 7% Obtusifoliol 26% Lanosterol
erg3Δ::LEU2 erg11::URA3	84% 14-Methylfecosterol 3% Obtusifoliol 12% Lanosterol
sld1-1	39% Ergosta-7,22-dien-3β-ol 20% Ergosta-8,24(28)-dien-3β-ol 20% Ergosta-7-en-3β-ol 8% Ergosta-7,24(28)-dien-3β-ol 7% Ergosta-8-en-3β-ol 4% Zymosterol 1% Ergosta-8,22-dien-3β-ol

[a]Cells were grown in yeast extract for 48 h, centrifuged and saponified as described previously (26). Gas chromatography and mass spectrometry analysis were performed as described in Materials and Methods. See Table 1 for abbreviation. Trace, much less than 1%.

sterol profile as that of JR4, with the exception that no ergosterol was detected. Thus, viability of JR4 is not the result of leakiness of the individual point mutations. Strain SG1, which has been previously identified as containing point mutations in the *ERG3* and *ERG11* genes (17), produced a sterol profile consistent with that of an *erg3* strain. Our isolate must have resulted from a spontaneous revertant of the *erg11* lesion.

Table 2 also shows the sterol profile of strain *sld1-1*, a strain which contains a wild-type copy of *ERG11*. This *sld1-1* marker originally arose in an aerobically growing variant of the *ERG11* disrupted strain, strain *erg11::URA3 sld1*. Since *erg11::URA3 sld1* was shown to retain its original *erg11::URA3* disruption based on uracil prototrophy, its ability to grow aerobically was attributed to a suppressor mutation at a different locus. The strain was mated to *erg11::URA3*, an obligate anaerobe, in order to determine whether the suppressor mutation was dominant or recessive. The resulting diploid was found to be also an obligate anaerobe, indicating that the *sld* mutation is recessive. Mating of *erg11::URA3 sld1* with an *ERG11 ura3-52* strain yielded a diploid for which sporulation resulted in a high percentage of incomplete tetrads. This may be due to the accumulation of decreased sterol levels or aberrant sterols in the developing asci. Of the four intact tetrads recovered, one (nonparental ditype) yielded 2:2 segregation for uracil prototrophs that were obligate anaerobes and uracil auxotrophs that were capable of aerobic growth. Thus, the *erg11* marker and the *sld1* marker are not linked. This aerobically competent *sld1* segregant was named *sld1-1* (Table 1). Sterol analysis of *sld1-1* yielded a spectrum that was consistent with that of an *erg3* strain (Table 2). Strain *sld1-1* was then mated separately with known *erg3* strains. The resulting diploids remained nystatin resistant, indicating that *erg3* and *sld1-1* are allelic (27).

PCR primers chosen for amplification of *ERG3* gave a single product of the predicted size using *ERG3* genomic DNA. When these identical amplimers were used with *sld1-1* genomic DNA as the template, a single band of the same 2.2 kb size was produced. Since this *sld1-1* strain was never shown to revert (27), it is of interest that *sld1* yielded a product that is the same as wild-type product, indicating no gross insertion or deletion has occurred.

As is the case with many of the *erg* mutants (11-13), *erg3* and *sld1-1* are cycloheximide sensitive, apparently due to the increased permeability of the cytoplasmic membrane. To further examine the *erg3* nature of *sld1-1*, we examined the capacity of the amplified gene sequence for either *ERG3* or *sld1-1* to restore cycloheximide resistance to an *erg3* null mutant. Plasmids were prepared containing either the amplified *ERG3* or *sld1-1* sequence under regulation of the galactose promoter and were transformed into strain *erg3Δ::LEU2*. The results are shown in Table 3. When the *ERG3* plasmid was present, cycloheximide resistance returned in the cells grown on YMG, but not in cells grown in YMM. However, addition of the *sld1-1* containing plasmid had no effect on cycloheximide sensitivity of cells grown on either medium. These patterns are consistent with *sld1* being a nonfunctional *erg3* allele.

The demonstration that the *erg11* lesion is not aerobically viable in *S. cerevisiae* (16), coupled with the results showing that azole-resistant mutants of this organism were *erg3* mutants, led to the hypothesis that the *erg11* mutation was lethal due to the formation of a sterol diol (19). Since azoles are inhibitors of C-14 demethylation (28), they are mimics for the *erg11* mutation. Thus, the *erg3* mutation would allow aerobic survival in the presence of azole *via* the same mechanism that allows survival of the *erg11* mutation when accompanied by the *erg3* mutation. The situation in the pathogenic fungus *C. albicans*, appears different in that *erg11* mutants are capable of aerobic growth. These mutants showed decreased growth rates (29), altered membrane properties (30) and defective hyphae formation (29). Coupled with recent results (31) indicating the increased active oxygen sensitivity of azole-treated cells, the mode of efficacy of these drugs appears quite complex.

TABLE 3

Cycloheximide Sensitivity of Strain LB4-3A *(erg3Δ::LEU2)* Transformed with Polymorase Chain Reaction Products Under the Galactose Promoter

Growth conditions[a]	Cycloheximide [Minimal inhibitory concentration (ng/mL)[b]					
Galactose	0	10	20	40	80	160
pGal (control)	+	+	+	−	−	−
pGal-*sld1*	+	+	+	−	−	−
pGal-ERG3	+	+	+	+	+	+
Glucose	0	10	20	40	80	160
pGal (control)	+	+	+	−	−	−
pGal-*sld1*	+	+	+	−	−	−
pGal-*ERG3*	+	+	+	+	−	−

[a]Cells were grown on yeast extract peptone media containing either 2% galactose plus 0.1% glucose, or 2% glucose.
[b]Results presented are a compilation of six independent clones of either *ERG3* or *sld1* gene sequences transformed into *erg3Δ::LEU2*; +, growth at 48 h; −, no growth at 48 h.

We previously reported the sterol profile of an *erg11* mutant (strain D10) of *C. albicans* (32). The sterols identified for D10 were 14-methylfecosterol, obtusifoliol, lanosterol, 24-methylene-24,25-dihydrolanosterol and 24,25-dihydrolanosterol. In view of the identification of a sterol diol in another *C. albicans* strain (20) and in the context of this study, the sterol content of D10 was reexamined. The sterol profile of D10 along with that of the *S. cerevisiae* strain *erg3Δ::LEU2 erg11::URA3* are shown in Figure 1. The *S. cerevisiae* strain shows the profile of C-14 methylsterols typically found in *erg11* strains in an *erg3* background. In the *C. albicans erg11* strain, a somewhat different array of C-14 methylsterols is seen to accumulate. The peak eluting at 18:03 has now been identified as 14α-methylergosta-8,24(28)-dien-3β,6α-diol rather than 24,25 dihydrolanosterol.

The MS analysis of the strain D10 diol yielded results similar to those reported in the identification of the diol in another *erg11* of this organism (20). The electron impact mass spectrum of this sterol showed the following ions: *m/z* 428, $M^{+\cdot}$; *m/z* 413, $M^{+\cdot} - \cdot CH_3$; *m/z* 395, $M^{+\cdot} - \cdot CH_3 - H_2O$; and *m/z* 377, $M^{+\cdot} - \cdot CH_3 - 2\ H_2O$. The formation of the trimethylsilyl derivatives using BSTFA (1% TMCS) resulted in a *bis*-trimethylsilyl derivative, which is consistent with the presence of a diol. The electron impact spectrum of this derivative yielded the following ions: *m/z* 572, $M^{+\cdot}$; *m/z* 557, $M^{+\cdot} - \cdot CH_3$; *m/z* 467, $M^{+\cdot} - \cdot CH_3 - HOSiMe_3$; and *m/z* 377, $M^{+\cdot} - \cdot CH_3 - 2\ HOSiMe_3$.

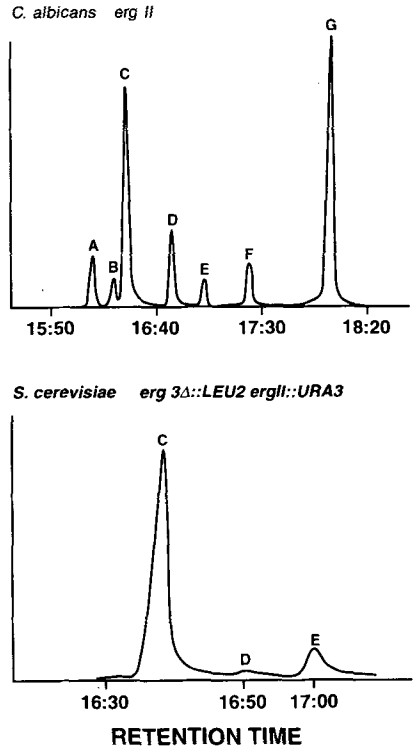

FIG. 1. Sterol profiles of *Candida albicans* strain D10 and *Saccharomycea cerevisiae* strain *erg11::URA3 erg3Δ::LEU2*. A, unidentified sterol of *m/z* 408; B, unidentified sterol of *m/z* 410; C, 14-methylfecosterol; D, obtusifoliol; E, lanosterol; F, 24-methylene lanosterol; G, 14-methylergosta-8,24(28)-dien-3β,6α-diol.

The results presented here indicate conclusively that, in the absence of ergosterol (detection level much less than 1%), removal of the methyl group from the C-14 position is not required for the aerobic growth of *S. cerevisiae*. Previous reports using leaky strains (JR4 and SG1) that produced low levels of ergosterol made a definitive determination regarding the requirement for C-14 demethylation impossible. This conclusion is clearly evident by the aerobic growth of *erg3Δ::LEU2 erg11::URA3*, a strain that produces 14-methylsterols exclusively. The results using *erg3* disrupted strains, and *erg11* suppressor strains (*sld1*) also confirm the necessity of an *erg3* mutation for the aerobic growth of *erg11* mutants of this organism. This confirmation supports the hypothesis that the *erg11* mutation is lethal due to the accumulation of sterol diol. The viability of *erg11* isolates from *C. albicans* and the detection of significant levels of diol indicate either that the two organisms produce differing amounts of diol or that they vary in their sensitivity to the lethal effects of the diol. In addition, the possibility exists that *C. albicans* may be spared the lethal effects of the diol by sequestering this compound in an esterified form.

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Farnesylamine: An Inhibitor of Farnesylation and Growth of *Ras*-Transformed Cells

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Farnesylamine, an analogue of farnesol, was shown to inhibit growth of PAP2 cells (*ras*-transformed NIH 3T3 cells) in a dose-dependent manner. This inhibition was overcome by adding farnesol to the culture medium, but not by adding geranylgeraniol, squalene, cholesterol, dolichol, myristic acid or palmitic acid. Farnesylamine inhibited both farnesyl/protein transferase and geranylgeranyl/protein transferase in whole cell extracts and also inhibited the prenylation of proteins, particularly *ras* p21, in PAP2 cells. Inhibition of prenylation was associated with increased biosynthesis of other products of the mevalonate biosynthetic pathway. These observations suggest that inhibition of the growth of PAP2 cells by farnesylamine may be due to blocking of *ras*-mediated signal transduction. This offers a means of investigating mechanisms involved in *ras* action and raises the possibility of developing novel strategies for anticancer therapy.

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Recently it has been established that polyisoprenoid compounds such as farnesol and geranylgeraniol are attached posttranslationally to a small group of proteins (1-4). Some of these proteins, such as *ras* proteins, are biologically active only when they are associated with the inner surface of plasma membranes (5-8). Several posttranslational modifications are required for interaction of these proteins with the plasma membrane. These include addition of a farnesyl group to the C-terminal cysteine residue by a thioether linkage (9,10), proteolytic cleavage of three amino acids at the C-terminal end, methylation of the carboxyl group of cysteine (11,12) and palmitoylation (13-15). A list of well-characterized farnesylated and geranylgeranylated proteins is included in recent reviews (7,16). Among these prenylated proteins, much attention has been focused on *ras* proteins, because of their involvement in many types of cancer (7,17-19).

Several reports suggest that both membrane association and biological activity of *ras* proteins are abolished by blocking farnesylation of these proteins. For example, altering the amino acid sequence at the C-terminal end of the *ras* protein to prevent farnesylation abolishes its cell transforming activity (20-22). The growth of *ras*-transformed cells and other cells can also be prevented by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which block the synthesis of mevalonic acid, a precursor of farnesyl pyrophosphate (FPP) (17,23,24). However, it was recently shown that inhibition of cell growth by lovastatin (an HMG-CoA reductase inhibitor) is independent of *ras* function (25), and the concentrations of such inhibitors required to prevent the prenylation of *ras* proteins in cell culture are approximately 100 times higher than the concentrations required to inhibit cholesterol biosynthesis (26). Similarly, high concentrations (1 mM) of *d*-limonene and its metabolites are required to prevent *ras* prenylation (27).

The enzymes involved in the transfer of farnesol and geranylgeraniol moieties from their pyrophosphate forms to protein have been identified in several mammalian cells and tissues (28-35). Farnesyl/protein transferase has been purified from rat brain (30), and a number of synthetic tetrapeptides were found to be good inhibitors of this enzyme *in vitro* (36). Biological activity of *ras* proteins has been inhibited in *Xenopus* oocytes by microinjection of an octapeptide with sequences similar to the C-terminal end of *ras* p21 protein (37). However, doubts were expressed about the use of these peptides as anticancer drugs, because their delivery to intracellular targets may present problems (19). Therefore, compounds that specifically inhibit farnesylation at low concentrations without affecting the biosynthesis of other compounds may be useful in controlling malignancies.

In this paper, we describe the synthesis of farnesylamine, a derivative of farnesol, and its use an inhibitor of cell growth and farnesylation of proteins in *ras*-transformed PAP2 (*ras*-transformed NIH 3T3) cells. In these cells, p21 *ras* levels are expressed about 100 times higher than in the NIH 3T3 cells from which they are derived (38).

MATERIALS AND METHODS

Materials. Mevinolin was a gift from Murray Huff (Department of Medicine, University of Western Ontario, London, Ontario, Canada). The lactone form of mevinolin was converted to its sodium salt as described by Habenicht *et al.* (39). (*RS*)[5-^{3}H]Mevalonolactone (MVA) (27.5 Ci/mmol) was obtained from New England Nuclear, Dupont Canada Inc. (Markham, Ontario, Canada). [^{3}H]-Thymidine (6.7 Ci/mmol) was from ICN (Irvine, CA). [1(n)-^{3}H]FPP (15 Ci/mmol) and [1(n)-^{3}H]geranylgeranyl pyrophosphate (GPP) (15 Ci/mmol) were purchased from Amersham Life Sciences (Oakville, Ontario, Canada). Farnesol was purchased from Sigma Chemical Co. (St. Louis, MO), and farnesyl bromide from Aldrich Chemical Co., Inc. (Milwaukee, WI). Tissue culture medium and fetal calf serum were purchased from GIBCO (Burlington, Ontario, Canada). X-Omat AR film was obtained from Eastman Kodak Co. (Rochester, NY). Electrophoresis reagents were from Bio-Rad (Richmond, CA). All other chemicals were purchased from Sigma.

Synthesis of farnesylamine. All-*trans* farnesylamine was synthesized from all-*trans* farnesol by the Gabriel synthesis (40). Farnesol was first converted to farnesyl tosylate and then to farnesylphthalamide, which was subjected to hydrazinolysis to yield the amine. Farnesylamine

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Abbreviations: DNA, deoxyribonucleic acid; FPP, farnesylpyrophosphate; GPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; MTT, 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide; MVA, mevalonic acid; PAP2 cells, *ras*-transformed NIH 3T3 cells; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TLC, thin-layer chromatography.

was separated from the reaction mixture and purified by silica gel 60 H thin-layer chromatography (TLC). Chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) was used as developing solvent, and the farnesylamine was detected by spraying with ninhydrin. Farnesylamine has an R_f of 0.58. The structure was confirmed by mass spectroscopy and nuclear magnetic resonance spectroscopy. All-*trans* farnesylamine was also synthesized in better yield from all-*trans* farnesyl bromide according to Galat and Elion (41).

Cell culture. PAP2 cells (malignant, T24 H-*ras*-transformed NIH 3T3 cells) (42,43) were maintained at 37°C in Dulbecco's modified Eagle medium containing 3.7 g of $NaHCO_3$ per liter, supplemented with 10% (vol/vol) fetal bovine serum. The medium was equilibrated with a humidified atmosphere of 5% CO_2. Stock cultures were seeded at a density of 2×10^5 cells/mL and allowed to multiply for 48 to 72 h. Viability of the cells was measured before and after treating with farnesylamine, using the 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide method as described by Hansen *et al.* (44). Cell proliferation was measured by plating cells in plastic dishes, allowing cell growth for the times specified, trypsinizing the cells and counting them with a hemacytometer.

Incorporation of [^{3}H]thymidine into DNA. PAP2 cells were plated at 1×10^4 cells/well in 96-well, flat-bottomed culture plates in a total volume of 200 μL of medium and incubated at 37°C for 24 h with or without various test compounds. [^{3}H]Thymidine (0.5 μCi/well) was then added, and after 4 h the cells were harvested onto a glass fiber filter paper using a semiautomatic 12-well cell harvester (Skatron Inc., Sterling, VA). Radioactivity on the filter paper was counted using ScintiVerse (Fisher Scientific, Napean, Ontario, Canada) in a liquid scintillation counter.

Incorporation of [^{3}H]MVA into proteins. Cells were cultured for 48 h, and 30 μM mevinolin, 100 μM unlabelled MVA and 50 to 100 μCi/mL (*RS*) [5-^{3}H]MVA (27.5 Ci/mmol) were added. Farnesylamine (20 μM when used) was also added at this time. After 24 h, the cells were trypsinized and collected by centrifugation at 500 × *g* for 5 min. They were washed with citrate saline or phosphate buffered saline (PBS) and used for different experiments.

For electrophoresis, cell pellets were dispersed in Tris-HCl, pH 7.4, and homogenized in the same buffer. The protein was precipitated with cold 10% trichloroacetic acid (TCA), and the precipitate was collected by centrifugation. It was washed twice with cold 10% TCA, then delipidated by extracting twice with diethyl ether. The precipitate was dissolved by heating at 100°C for 5 min in a sample buffer containing 8M urea, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 0.0625 M Tris-HCl, pH 6.8. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (45). After staining with Coomassie Blue, the gels were treated with fluorographic enhancer, En^{3}Hance, dried and exposed to X-OMAT AR film for 20 d at −80°C.

TCA precipitation. After labelling the cells with [^{3}H]MVA, they were washed with PBS and homogenized in a buffer containing 0.02 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 M NaCl, 1% vol/vol Triton X-100, 0.5% SDS and 3 mM phenylmethylsulfonylfluoride (PMSF), and centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant was used for TCA precipitation and immunoprecipitation. Equal amounts of protein were taken and precipitated with 30% TCA and 4% SDS and filtered through micro-glass filters as described by Reiss *et al.* (30).

Immunoprecipitation. To 800 μg of protein in 1-mL buffered solution, were added 10 μL of an anti-v-H-*ras* monoclonal antibody (Ab-1; clone Y13-259) (Oncogene Science, Manhasset, NY) and 15 μL goat anti-rat IgG/protein A *Staphylococcus* complex. The mixture was incubated at 4°C for 24 h with a rocking device. The precipitate was collected by centrifugation in a microcentrifuge at 2500 rpm for 15 min and washed four times with PBS, ph 7.25, containing 1% Triton X-100, 0.5% deoxycholate and 0.1% SDS, dissolved in ScintiVerse (Fisher Scientific) and counted in a liquid scintillation counter.

Farnesyl/protein and geranylgeranyl/protein transferase assays. Farnesyl/protein transferase assay was carried out essentially as described by Reiss *et al.* (30). [^{3}H]FPP (300,000 dpm) and various amounts of farnesylamine were mixed and dried under nitrogen. PAP2 cells (at about 80% confluency) were treated with 30 μM mevinolin for 3 h and harvested. The cells were washed with PBS several times and homogenized in a buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.2% β-octaloglucoside, 2 mM dithiothreitol, 5 mM $MgCl_2$ and 100 mM NaF. Cell debris was pelleted by low-speed centrifugation. The supernatant was used as enzyme source. Cell extract (100 μL; 40 μg of protein) was added to the mixture of labelled FPP and farnesylamine and incubated for 1 h at 30°C. The reaction was stopped by adding 0.5 mL of 30% TCA and 0.5 mL of 4% SDS and processed as described by Reiss *et al.* (30). Similarly, geranylgeranyl/protein transferase assays were carried out by using [^{3}H]GPP instead of [^{3}H]FPP.

Biosynthesis of other isoprenoid compounds in presence of farnesylamine. After labelling the cells with [^{3}H]MVA as described above, they were washed with PBS several times and homogenized in a glass homogenizer with acetone. The resulting mixture was centrifuged, and the pellet was washed three times with chloroform/methanol (2:1, vol/vol), three times with chloroform/methanol/water (10:10:3, by vol) and finally three times with diethyl ether. All the washings were pooled, dried and saponified for 6 h with NaOH in presence of methanol. The nonsaponified lipids were extracted with ether and washed with water, and the lipids were applied on reverse-phase TLC and developed with acetonitrile/water (9:1, vol/vol). Areas corresponding to geraniol, farnesol and geranylgeraniol were scraped off and counted. The radioactive material at the origin was scraped off, extracted and applied onto a silica gel 60H TLC and developed with *n*-hexane/diethyl ether/acetic acid (65:35:1, by vol). The areas corresponding to cholesterol, dolichol and coenzyme Q were scraped off and counted.

RESULTS

Effects of farnesylamine and other compounds on the growth of PAP2 cells in culture. Proliferation of PAP2 cells was significantly inhibited by farnesylamine at a concentration of 7.5 μM, as measured by incorporation of thymidine into DNA (Fig. 1A) as well as by cell growth studies (Fig. 1B). Cells were viable at concentrations of farnesylamine up to 50 μM. Farnesol and farnesyl bromide

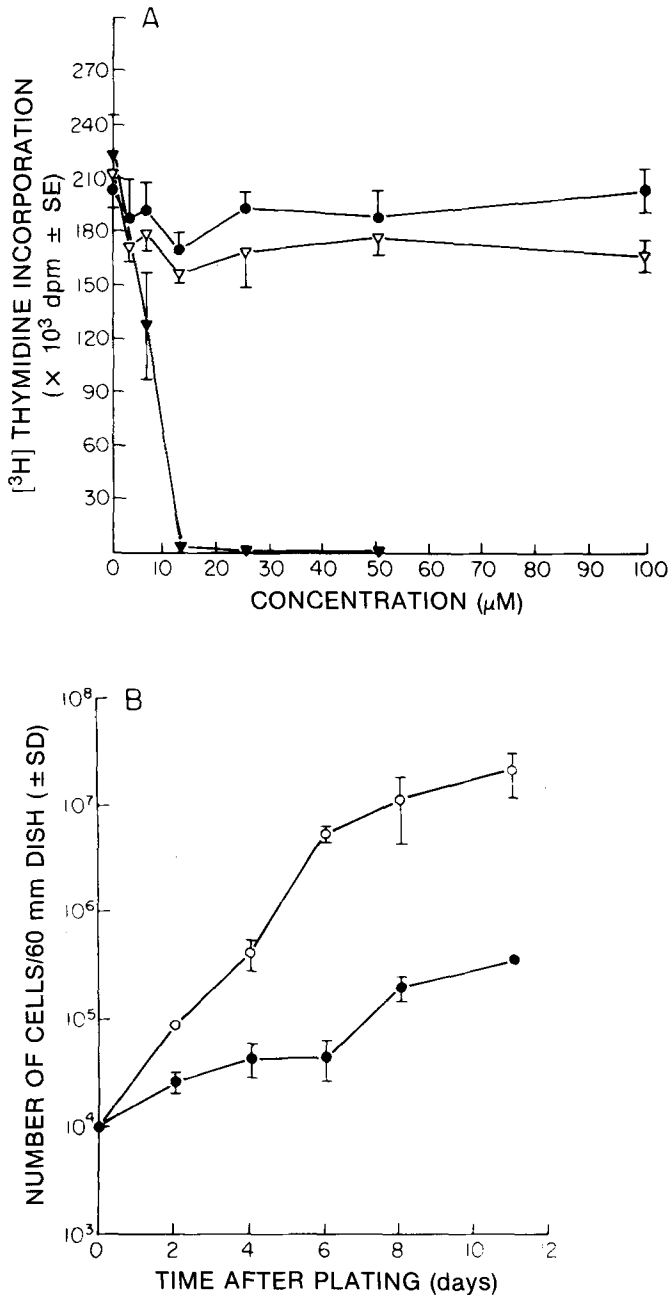


FIG. 1. A: Effects of farnesol, farnesyl bromide and farnesylamine on *ras*-transformed NIH 3T3 (PAP2) cells. Cells were plated in triplicate at 2 × 10^4 cells/well in 96-well, flat-bottomed, culture plates. Different concentrations of farnesol (●), farnesyl bromide (▽) or farnesylamine (▼) were added as indicated in the figure, and the incorporation of [^{3}H]thymidine was measured. Points are the average of mean values from three experiments ± SE. B: Growth of PAP2 cells in presence (●) or absence (○) of 7.5 μM farnesylamine. Cells were plated in triplicate in 60-mm^2 culture dishes. Cells were removed by trypsinization at specified times and were counted by a hemocytometer. Points are mean values ± SD.

did not inhibit cell growth at concentrations up to 100 μM (Fig. 1A). Various straight-chain, saturated amines-(C_6-C_{18}) were also tested with PAP2 cells. Short-chain amines (C_6, C_8) did not inhibit cell growth, whereas longer-chain amines inhibited at higher concentrations (IC_{50} 12.5 to 50 μM) (Kothapalli, R., Lui, E.M.K., Guthrie, N., Chambers, A.F., and Carroll, K.K., unpublished data).

TABLE 1

Effect of Various Compounds on the Growth of Farnesylamine Treated Cells[a]

Compounds added	[^{3}H]Thymidine incorporation into DNA (× 10^3 dpm ± SD)
Control	227.7 ± 23
Farnesylamine	4.2 ± 0.9
+ Farnesol	215.8 ± 26
+ Geranylgeraniol	3.1 ± 0.6
+ Squalene	5.2 ± 0.8
+ Cholesterol	4.7 ± 0.4
+ Dolichol	4.3 ± 0.5
+ Myristic acid	4.5 ± 0.3
+ Palmitic acid	5.8 ± 0.7

[a]*ras*-Transformed NIH 3T3 cells were cultured with or without 10 μM farnesylamine for 24 h. Other compounds (20 μM) were added to the cells treated with farnesylamine, and the incorporation of [^{3}H]thymidine into DNA was measured after 14 h.

The growth inhibition of PAP2 cells by farnesylamine was overcome by adding farnesol to the culture medium, but not by adding various other isoprenoids or fatty acids (Table 1). Inhibition of cell proliferation by various other long-chain amines was not overcome by adding farnesol (Table 2).

Mechanism of action of farnesylamine. The mechanism by which farnesylamine inhibits cell growth was investigated by labelling PAP2 cells with [^{3}H]MVA in the presence or absence of farnesylamine. Proteins were extracted, subjected to SDS-PAGE and exposed to x-ray film. Inhibition of prenylation of proteins was observed in cells treated with farnesylamine (Fig. 2).

In order to determine whether farnesylamine inhibits the transfer of farnesyl moieties from FPP to protein, extracts of PAP2 cells were prepared and incubated with [^{3}H]FPP plus different concentrations of farnesylamine. Assays for farnesyl/protein transferase activity showed

TABLE 2

Inability of Farnesol to Reverse Inhibition of Cell Proliferation by Long-Chain Fatty Amines[a]

Amines added	[^{3}H]Thymidine incorporation into DNA (× 10^3 dpm ± SD)
Control (without any fatty amines)	125.7 ± 13.8[b]
Dodecylamine (25 μM)	29.7 ± 2.1
Dodecylamine + farnesol	28.2 ± 2.8
Tridecylamine (12.5 μM)	20.5 ± 1.8
Tridecylamine + farnesol	21.2 ± 1.3
Tetradecylamine (12.5 μM)	30.1 ± 4.3
Tetradecylamine + farnesol	25.4 ± 0.52
Hexadecylamine (25 μM)	25.3 ± 1.3
Hexadecylamine + farnesol	27.1 ± 2.8
Octadecylamine (50 μM)	48.1 ± 1.2
Octadecylamine + farnesol	47.0 ± 5.6

[a]*ras*-Transformed NIH 3T3 cells were cultured with or without various long-chain amines (dissolved in dimethyl sulfoxide) for 24 h. Farnesol (20 μM) was added to the cells at that time, and the incorporation of [^{3}H]thymidine into the DNA was measured after 14 h.
[b]This value is dpm ± SE rather than SD.

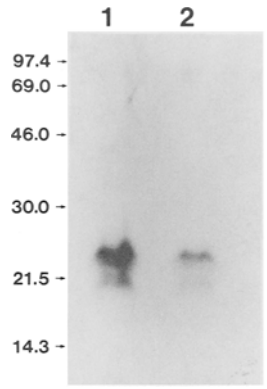


FIG. 2. Electrophoretic profiles of farnesylated proteins in *ras*-transformed NIH 3T3 cells. Lane 1: 160 μg of total protein obtained from control cells (without farnesylamine). Lane 2: 130 μg of total protein obtained from cells treated with 20 μM of farnesylamine.

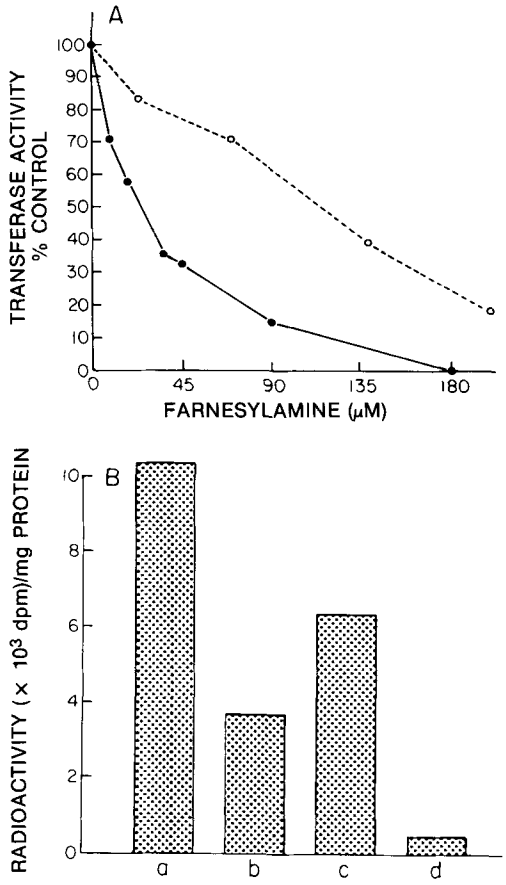


FIG. 3. A: Inhibition of farnesyl/protein transferase and geranylgeranyl/protein transferase in cell extracts by farnesylamine. Details of the assays are given in the text. B: Effect of farnesylamine on the synthesis of farnesylated proteins in intact cells. *ras*-Transformed NIH 3T3 cells were labelled with [^{3}H]mevalonic acid, and total proteins were extracted and precipitated with trichloroacetic acid (TCA) or with antibodies raised against *ras* protein. a, Total TCA precipitable protein in control; b, total TCA precipitable protein in farnesylamine-treated cells; c, immunoprecipitable protein in control cells; d, immunoprecipitable protein in farnesylamine-treated cells.

TABLE 3

Biosynthesis of Other Isoprenoid Compounds in Presence or Absence of Farnesylamine in the Medium[a]

Compound	Control (dpm/100 mg of cells)	Farnesylamine treated (dpm/100 mg of cells)
Cholesterol	26,501	37,792
Dolichol	1,387	1,841
Coenzyme Q	1,095	1,291

[a]*ras*-Transformed NIH 3T3 cells were labelled with [^{3}H]mevalonic acid. After 16 h, total lipids were extracted and saponified. The non-saponified lipids were extracted, and the amount of radioactivity incorporated into various compounds was measured as indicated in the Materials and Methods section.

that the farnesyl moiety was transferred from FPP to proteins and that the transfer was inhibited by farnesylamine. Farnesylamine was similarly shown to inhibit geranylgeranyl/protein transferase activity (Fig. 3A).

To confirm that farnesylamine affects the formulation of farnesylated proteins in intact cells, particularly *ras* proteins, PAP2 cells were labelled with [^{3}H]MVA in the presence or absence of farnesylamine, and total proteins were extracted. A portion of the extracted proteins was precipitated with TCA and filtered through a glass fiber filter. The remaining portion was immunoprecipitated by anti-v-H-*ras* monoclonal antibody (Ab-1, clone Y13-259). The amount of radioactivity incorporated into total protein and *ras* proteins was determined. Prenylation of total proteins was inhibited by 60% and prenylation of *ras* protein by 90% in farnesylamine-treated cells (Fig. 3b).

Effect of farnesylamine on biosynthesis of other isoprenoid compounds. Increased synthesis of cholesterol, dolichol and coenzyme Q was observed in cells treated with farnesylamine compared to untreated cells (Table 3).

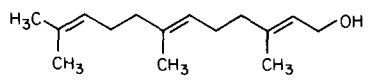
DISCUSSION

Analogues of purines have been prepared and successfully used as anticancer drugs (46). As part of a similar strategy, we synthesized and tested farnesylamine, an amino analogue of farnesol (Scheme 1).

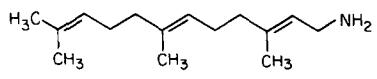
Farnesylamine inhibited the growth of PAP2 cells more effectively than various straight-chain, saturated fatty amines. The inhibition by farnesylamine could be overcome by farnesol, but not by other isoprenoids or fatty acids (Table 1). This suggested that farnesylamine was acting by specifically interfering with farnesylation of proteins, particularly *ras* protein. The inhibition of cell growth by other amines could not be overcome by farnesol, and they may be acting by some other mechanism, such as inhibition of protein kinase C (47).

Our experiments showed that farnesylamine inhibits farnesyl/protein transferase, which catalyzes the transfer of farnesol from FPP to the protein (Fig. 3A). Further experiments also showed that prenylation of *ras* protein by PAP2 cells was inhibited to a greater extent than prenylation of the cellular proteins as a whole (Fig. 3B).

Farnesylamine also inhibited geranylgeranyl/protein transferase *in vitro* (Fig. 3A). Farnesyl and geranylgeranyl transferases have a common α-subunit for substrate binding (48), so farnesylamine may be inhibiting both enzyme activities by interacting with that subunit. It is possible that inhibition of geranylgeranylation of proteins may be



FARNESOL



FARNESYLAMINE

SCHEME 1

partly responsible for the growth inhibition by farnesylamine. However, the fact that the inhibition could be overcome by farnesol and not by geranylgeraniol suggests that inhibition of farnesylation may be a more important factor. It should also be noted that *ras* protein is greatly overexpressed in PAP2 and is probably largely responsible for the increased growth potential of these cells.

The FPP that serves as a substrate for farnesylation of proteins by farnesyl/protein transferase is most probably derived from the mevalonate biosynthetic pathway. If less of it is utilized for farnesylation, more will be available for conversion to other products of the pathway. This could account for the increased biosynthesis of cholesterol, dolichol and coenzyme Q in PAP2 cells treated with farnesylamine compared to controls (Table 3).

Overall, these results suggest that inhibition of the growth of PAP2 cells by farnesylamine is due primarily to inhibition of farnesylation of proteins, particularly *ras* p21, with farnesylamine acting as an inhibitor of farnesyl/protein transferase. This provides a means of investigating the role of *ras* in cellular metabolism and suggests a novel approach to controlling *ras*-mediated malignant cell growth.

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Phospholipid and Fatty Acid Compositions of *Rhizobium leguminosarum* biovar *trifolii* ANU843 in Relation to Flavone-Activated pSym *nod* Gene Expression

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The phospholipid and associated fatty acid compositions of the bacterial symbiont of clover, *Rhizobium leguminosarum* biovar *trifolii* wild-type ANU843, was analyzed by two-dimensional silica thin-layer chromatography, fast atom bombardment–mass spectrometry, flame-ionization detection gas–liquid chromatography and combined gas–liquid chromatography/mass spectrometry. The phospholipid composition included phosphatidylethanolamine (15%), *N*-methylphosphatidylethanolamine (47%), *N,N*-dimethylphosphatidylethanolamine (9%), phosphatidylglycerol (19%), cardiolipin (5%) and phosphatidylcholine (2%). Fatty acid composition included predominantly *cis*-11-octadecenoic acid, lower levels of *cis*-9-hexadecenoic acid, hexadecanoic acid, 11-methyl-11-octadecenoic acid, octadecanoic acid, 11,12-methyleneoctadecanoic acid, eicosanoic acid and traces of branched, and di- and triunsaturated fatty acids. The influence of expression of the "nodulation" genes encoding symbiotic functions on the composition of these membrane lipids was examined in wild-type cells grown with or without the flavone inducer, 4′,7-dihydroxyflavone and in mutated cells lacking the entire symbiotic plasmid where these genes reside, or containing single transposon insertions in selected nodulation genes. No significant changes in phospholipid or associated fatty acid compositions were detected by the above methods of analysis.

Lipids 28, 975–979 (1993).

The initiation of the nitrogen-fixing root-nodule symbiosis between *Rhizobium* and its legume host involves extensive bilateral signaling. In response to plant-excreted flavonoids, several nodulation (*nod*) genes residing on the large symbiotic plasmid (pSym) of *Rhizobium* are expressed, and their action fulfills pivotal roles in the establishment of the bacteria-plant symbiotic program. Several pSym Nod proteins are membrane-associated and are proposed to be involved in the synthesis of a family of acylated chitooligosaccharides (1–3). Although these glycolipids were initially isolated from culture supernatants and thus considered extracellular, their amphiphilic character argues that they reside primarily in bacterial membranes. Recently, we obtained evidence of the membrane association of such acylated glucosaminoglycans (Orgambide, G.G., Hollingsworth, R.I., and Dazzo, F.B., unpublished data). Furthermore, we previously reported the characterization of a membrane-associated diglycosyl diacylglycerol (BF-7) from *Rhizobium leguminosarum* biovar *trifolii* (*R. trifolii*) ANU843 (4), a wild-type strain used extensively in studies of the *Rhizobium*-clover symbiosis. In subsequent studies, we found that cellular accumulation of BF-7 was increased when ANU843 was grown in the presence of the *nod* gene inducer 4′,7-dihydroxyflavone (DHF) and reduced in a *nodA*::Tn*5* mutant derivative (5). Since the above *nod*-influenced glycolipids are all minor membrane components, we wondered whether flavone-activated expression of *nod* genes also affects the composition of the major class of membrane lipids, namely the phospholipids. An earlier study addressing this question concluded that *nod* gene expression does not influence the phospholipid composition of the alfalfa symbiont, *R. meliloti* (6). However, a recent preliminary report suggested that induction of *nodFE* gene expression in the pea symbiont, *R. leguminosarum* bv. *viciae*, resulted in *de novo* synthesis of phospholipids with specific polyunsaturated fatty acids (7).

In the present study, we examined the phospholipid and associated fatty acid compositions of *R. trifolii* ANU843 grown in defined medium, and evaluated the effect of alterations in pSym *nod* composition and of the *nod* gene inducer DHF on this major class of membrane lipids.

MATERIALS AND METHODS

Bacterial cultures and preparation of the lipid extract. Strains of *R. trifolii* used were wild-type ANU843, its pSym-cured derivative ANU845 and transposon insertion *nod* mutant derivatives of ANU843, including strains ANU851 (*nodD*::Tn*5*), ANU252 (*nodA*::Tn*5*), ANU258 (*nodE*::Tn*5*), ANU251 (*nodL*::Tn*5*), ANU896 (*nodM*::mud*lacZ*) and ANU895 (*nodN*::mud*lacZ*), all kindly provided by B. Rolfe, Australian National University, Canberra, Australia (see Ref. 8 for strain characteristics). Bacteria were grown aerobically at 30°C in defined BIII broth (9), with or without 4 μM DHF, and harvested by centrifugation in late exponential phase at a density of 7.5×10^8 cells/mL. The cell pellet was washed twice with water, extracted by stirring with 100 mL chloroform/methanol/water (2:1:0.5, by vol), for 20 h at 25°C under a nitrogen atmosphere, and the organic extract was then filtered through a Millipore GVWP04700 (0.22 μm) membrane (Milford, MA). In comparison with a Bligh and Dyer extraction (10) from the same batch of ANU843 cells, this extraction procedure yielded the same pattern of phospholipids in silica thin-layer chromatography (TLC) but with a slightly higher recovery. The phospholipid extract represented approximately 10% (w/w) of the dry cell residue for all cultures. Phospholipid composition of transposon-insertion *nod* mutants was compared to ANU843 and ANU845 based on silica TLC of lipid extracts from

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Abbreviations: CL, cardiolipin; DHF, 4′,7-dihydroxyflavone; DMPE, *N,N*-dimethylphosphatidylethanolamine; FAB-MS, fast atom bombardment–mass spectrometry; FID-GLC, flame-ionization detection gas–liquid chromatography; GLC/MS, gas–liquid chromatography/mass spectrometry; MPE, *N*-methylphosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; pSym, symbiotic plasmid; TLC, thin-layer chromatography.

cells grown at 30°C for 5 d on BIII plates containing 4 μM DHF and solidified with 1% purified agar.

Characterization of the phospholipid components. Phospholipid extracts were analyzed by fast atom bombardment-mass spectrometry (FAB-MS) using a JEOL HX-110 mass spectrometer (JEOL USA, Peabody, MA) at an accelerating voltage of 10 kV, and *m*-nitrobenzyl alcohol and triethanolamine as matrices for positive and negative mode analyses, respectively. Individual phospholipid classes were purified from the lipid extract of ANU843 cells by two-dimensional TLC on silica plates (Whatman, Hillsboro, OR), preactivated for 1 h at 100°C, with chloroform/methanol/ammonia (70:35:5, by vol) as solvent for dimension I and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) for dimension II and characterized by FAB-MS. Fatty acid methyl ester derivatives were prepared by alkaline methanolysis (11) of an aliquot of the phospholipid extracts. Fatty acid derivatives were identified by gas-liquid chromatography/mass spectrometry (GLC/MS), using a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) fitted with a DB-1 column and coupled to a JEOL AX505H mass spectrometer (JEOL USA), by matching both their retention times and mass spectra to those of authentic standards. Quantification was by flame-ionization detection gas-liquid chromatography (FID-GLC), using a Hewlett-Packard 5890 instrument fitted with a DB-1 column.

Phospholipid quantitation. Silica TLC plates of phospholipid extracts were sprayed with either phosphomolybdic acid (phospholipid stain) or sulfuric acid/methanol (25:75, vol/vol, universal stain), followed by heating at 150°C. Densitometry was performed using a high-resolution charge-coupled device camera and an Ambis Optical Imaging System (San Diego, CA) with individual background subtraction for each spot. The relative abundance of each phospholipid class was calculated from densitometry values obtained from phosphomolybdic acid- and H_2SO_4-stained plates, corrected for the specific staining response of phospholipid standards. Values reported are an average from two independent batch cultures of ANU843 − DHF, ANU843 + DHF, and ANU845 + DHF.

RESULTS AND DISCUSSION

Phospholipid and fatty acid composition of R. trifolii *ANU843 grown without DHF.* Two-dimensional silica TLC of the phospholipid extract obtained from ANU843 separated several classes of phospholipid components (Fig. 1A). Negative mode FAB-MS analysis of each isolated lipid class led to the identification of *N*-methylphosphatidylethanolamine (MPE) (yielding quasimolecular ions $[M-H]^-$ at *m/z* 756 [predominant], 758, 770, 784, 798, daughter ion fragments formed by elimination of *N*-methyl-vinylamine [−57 amu], and low-mass diagnostic ions at *m/z* 154 and 194), and *N,N*-dimethylphosphatidylethanolamine (DMPE) (quasimolecular ions at *m/z* 744, 770 [predominant], 772, 784, daughter ion fragments formed by elimination of *N,N*-dimethyl-vinylamine [−71 amu] and low-mass diagnostic ions at *m/z* 168 and 208) (12). The analysis also revealed the presence of phosphatidylglycerol (PG) (quasimolecular ions at *m/z* 747, 773 [predominant], 775, 787, 789, 845, daughter ion fragments formed by elimination of dehydrated glycerol [−74 amu],

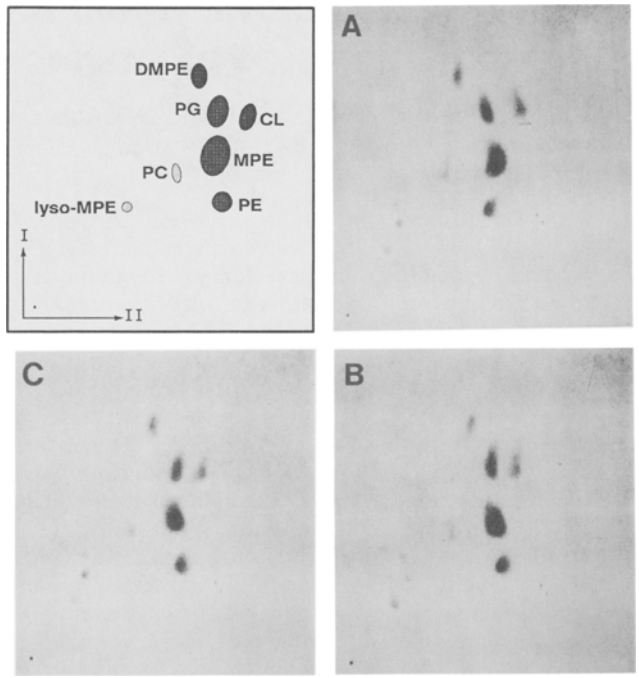

FIG. 1. Two-dimensional silica thin-layer chromatography analysis of phospholipid extracts from: (A) ANU843 wild-type grown without 4′,7-dihydroxyflavone (DHF), (B) ANU843 grown in presence of 4 μM DHF, (C) symbiotic plasmid-cured derivative ANU845 grown with 4 μM DHF (staining: 25% H_2SO_4 in methanol, 150°C). Abbreviations: DMPE, *N,N*-dimethylphosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; CL, cardiolipin; MPE, *N*-methylphosphatidylethanolamine; PE, phosphatidylethanolamine.

and low-mass diagnostic ions at *m/z* 153, 171 and 211), and cardiolipin (CL) (quasimolecular ions at *m/z* 1453, 1455, 1457, 1459, 1479, 1481, 1483, 1493, 1495, 1497, fragment ions produced by loss of the acyl glycerol portion of one phosphatidic acid group and/or elimination of fatty acyl residues, and prominent low-mass ions at *m/z* 137 [glycidol phosphate] and 153 [allyl phosphate]) (13). The identity of PG and CL was further verified by cochromatography on silica TLC of the phospholipid extract with PG or CL authentic standards. Phosphatidylethanolamine (PE) was identified as a fifth phospholipid in the extract based on a mass spectrum showing quasimolecular ions at *m/z* 676, 690, 702, 716, 718, 732, positive reaction to ninhydrin staining, and cochromatography with a standard. Only minute amounts of product were recovered from two minor TLC fractions, and their mass spectra yielded tentative assignments to phosphatidylcholine (PC) (also supported by cochromatography with a PC standard), and lyso-MPE. Upon positive mode FAB-MS analysis of the phospholipid extract, PE, MPE and DMPE species provided prominent molecular ions $[(M + H)^+$ and $(M + Na)^+]$, high-mass fragments and low-mass diagnostic ions consistent with the molecular species identified in negative mode, and with literature data (12,13), whereas the acidic species PG and CL were barely detected. The occurrence of PC (primarily as dioleoyl species) was confirmed by this positive mode FAB-MS analysis showing pseudomolecular ions $(M + H)^+$ and $(M + Na)^+$ at *m/z* 786 and 808, a fragment ion generated by loss of phosphocholine from $(M + H)^+$ at

m/z 603 (this connection between the two ions was verified by mass spectrometry/mass spectrometry analysis), and low-mass diagnostic ions at *m/z* 184 and 224 (12,13). Based on densitometry of the digitized TLC plate, the relative abundance of the different phospholipids in the ANU843 cell extract were: PC 2%, DMPE 9%, MPE 47%, PE 15%, PG 19%, CL 5.0%, and lyso-MPE 3%.

Alkaline methanolysis of the ANU843 phospholipid extract, followed by GLC/MS characterization and FID-GLC quantitation of the fatty acid methyl esters, revealed the large predominance of *cis*-11-octadecenoic acid (18:1), together with low levels of *cis*-9-hexadecenoic acid (16:1), hexadecanoic acid (16:0), 11-methyl-11-octadecenoic acid (11-Me-18:1; Ref. 14), octadecanoic acid (18:0), 11,12-methyleneoctadecanoic acid (19:0Δ) and eicosanoic acid (20:0) (Table 1). Minute amounts (<0.1%) of tetradecanoic acid (14:0), *iso*-pentadecanoic acid (*i*-15:0), *iso*-hexadecanoic acid (*i*-16:0), *anteiso*-heptadecanoic acid (*a*-17:0), *iso*-heptadecanoic acid (*i*-17:0), 9,10-methylenehexadecanoic acid (17:0Δ) and heptadecanoic acid (17:0) fatty acid methyl esters were also detected by FID-GLC. Negative mode FAB-MS analysis of the phospholipid samples also suggested the presence of trace amounts of several unusual fatty acids detected by their carboxylate anion at *m/z* 279 (18:2), 297 (19:0 or hydroxy-18:1), 305 (20:3), 307 (20:2), 309 (20:1), 325 (21:0 or hydroxy-20:1), 337 (22:1), and 339 (22:0 or hydroxy-21:1).

Effect of DHF and pSym on growth rate of R. trifolii *ANU843.* Previous studies showed that 4 μM DHF supplement to BIII broth activates the expression of pSym *nod* genes at least 10-fold higher than the constitutive background level in ANU843 (8,15). The exponential growth rates of ANU843 grown in BIII broth with or without 4 μM DHF, and the pSym-cured derivative ANU845 grown with 4 μM DHF, were 0.29, 0.36 and 0.46 generation/hour, respectively. These results indicate that DHF at 4 μM partially inhibits growth of ANU843 and that this inhibition is pSym-dependent.

Evaluation of the influence of DHF and pSym nod *genes on phospholipid and associated fatty acid compositions of* R. trifolii *ANU843.* The phospholipid extracts obtained from cells of standardized broth cultures of ANU843 grown with or without DHF, and from the pSym-cured derivative ANU845 grown with DHF, were compared by FAB-MS analysis, two-dimensional silica TLC with video densitometry quantitation and FID-GLC analysis of fatty acid methyl esters. These analyses did not reveal major changes in phospholipid or associated fatty acid composition of these three cultures (Figs. 1-3, Table 1), including the fatty acids detected in trace amounts by negative mode FAB-MS analysis as described above. A silica TLC survey of the polar lipid composition of mutant derivatives of ANU843 containing transposon insertions in selected *nod* genes (*nodD, nodA, nodE, nodL, nodM* or *nodN*) from the four operons in the 14 Kb *Hind*III pSym *nod* region (15) was also performed. When phosphomolybdic acid was used to detect phospholipids, all strains displayed the same qualitative and quantitative pattern (data not shown). However, a universal stain (25% H_2SO_4 in methanol, 150°C) revealed slight differences among minor lipids in the extracts from wild-type and *nod* mutants (Fig. 4). Since these minor lipids did not stain with phosphomolybdic acid (thus were unlikely to be phospholipid components), and were below detection by two-dimensional TLC, they were not investigated further in this study.

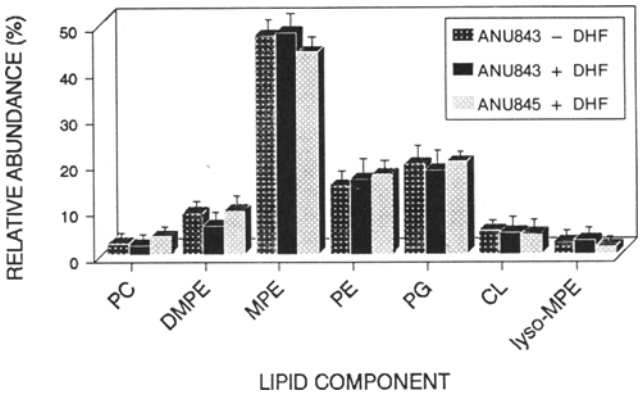

FIG. 2. Relative abundance of individual phospholipid classes in the lipid extracts from *R. trifolii* wild-type ANU843 grown without DHF (ANU843 − DHF), ANU843 grown in presence of 4 μM DHF (ANU843 + DHF), and pSym-cured derivative ANU845 grown with 4 μM DHF (ANU845 + DHF). The minor components from both ANU843 and ANU845 present in the lower left quadrant of the thin-layer chromatography plates (Fig. 1) were not included in this quantitative evaluation since the subtle variations in their relative intensity were not reproducible. See Figure 1 for abbreviations.

TABLE 1

Relative Abundance of Individual Fatty Acid Species in the Phospholipid Extracts from *R. trifolii* Wild-Type ANU843 Grown Without DHF (843-DHF), ANU843 Grown in Presence of 4 μM DHF (843 + DHF), and pSym-cured Derivative ANU845 Grown with 4 μM DHF (845 + DHF)

Phospholipid extract	Fatty acid (%)[a]						
	16:1	16:0	18:1	18:0	11-Me-18:1	19:0Δ	20:0
843 − DHF	1.0 (0.05)	3.3 (0.30)	81.0 (0.95)	11.0 (0.60)	1.1 (0.35)	2.4 (0.20)	0.2 (0.04)
843 + DHF	0.9 (0.25)	3.2 (0.30)	81.5 (1.15)	10.7 (0.35)	1.0 (0.05)	2.5 (1.10)	0.2 (0.07)
845 + DHF	0.9 (0.05)	3.2 (0.30)	82.1 (0.80)	10.4 (0.50)	1.1 (0.20)	2.0 (0.35)	0.3 (0.11)

[a]% of total fatty acids. Values in parentheses correspond to the standard error of the means. See text for full definition of each fatty acid. DHF, 4',7-dihydroxyflavone; pSym, symbiotic plasmid.

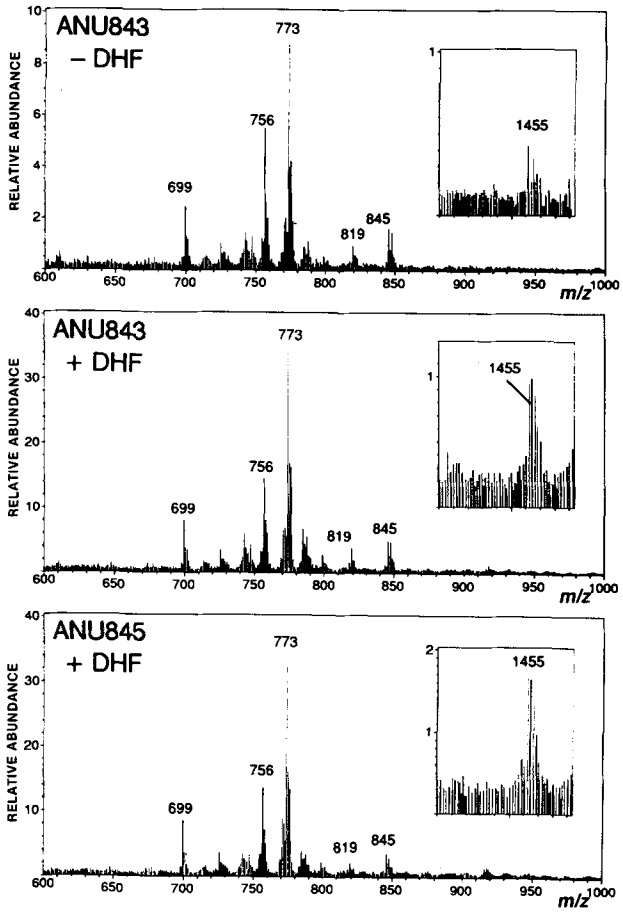

FIG. 3. Negative mode fast atom bombardment-mass spectrometry spectra of phospholipid extracts from ANU843 wild-type grown without DHF, ANU843 grown in presence of 4 μM DHF, and pSym-cured derivative ANU845 grown with 4 μM DHF (see text for ion assignments). See Figure 1 for abbreviations.

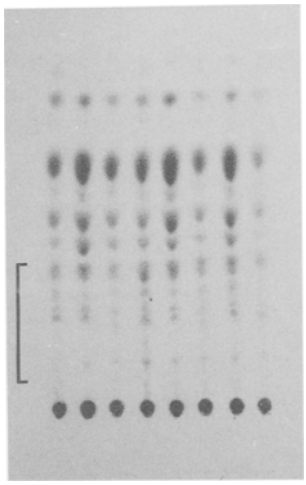

FIG. 4. One-dimensional thin-layer chromatography (TLC) analysis of phospholipid extracts from: (1) ANU843 wild-type, and various mutant derivatives (2) *nodD*::Tn*5*, (3) *nodA*::Tn*5*, (4) *nodE*::Tn*5*, (5) *nodL*::Tn*5*, (6) *nodM*::mud*lacZ*, (7) *nodN*::mud*lacZ*, (8) pSym-cured ANU845 (solvent: chloroform/methanol/ammonia, 70:35:5, by vol; staining: 25% H_2SO_4 in methanol, 150°C). Bracket indicates the area of the TLC where minor changes are observed.

The predominance of components of the PE series (PE, MPE, DMPE), together with significant amounts of PG and CL in *R. trifolii* ANU843, is consistent with data reported for certain other *Rhizobiaceae* isolates, including *R. meliloti* (6,16), the soybean symbiont *Bradyrhizobium japonicum* (17,18) and species of the plant pathogen *Agrobacterium* (19). In contrast, *R. loti* contained high amounts of phosphatidylserine and phosphatidylinositol (20), and an isolate of *R. leguminosarum* (bv. *viciae*) lacked MPE (21). A major difference in phospholipid composition between ANU843 and the other isolates of *Rhizobiaceae* reported is the level of PC (2% in ANU843, whereas it exceeds 20% in the other isolates). The relative level of PC in *A. tumefaciens*, as well as in *R. meliloti*, has been shown to increase with progression of cultures from exponential to stationary phase (16). The fact that *R. trifolii* ANU843 cells in the present study were harvested while in exponential rather than stationary phase might explain why they contained a low proportion of PC.

Several previous lines of evidence suggested that flavone-induced pSym *nod* expression modulates the accumulation of membrane-associated Nod proteins and minor membrane glycolipids. In the present study we addressed whether the phospholipids and their associated fatty acids were also altered when *Rhizobium* was grown to express its pSym *nod* genes. A second reason to test this possibility was our finding that ANU843 exhibits a pSym-dependent inhibition of growth in defined medium when DHF is added to activate *nod* expression. A combination of one- and two-dimensional TLC, FAB-MS, FID-GLC and GLC/MS analyses detected no significant difference in these membrane components between cultures of wild type *R. trifolii* ANU843 and its pSym-cured derivative ANU845 after at least seven generations in flavone induction medium. Thus, in wild-type ANU843, minor membrane glycolipids appear to be more central than phospholipids to the *nod*-encoded metabolism that enables the development of the *Rhizobium*-clover symbiosis.

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Age, Sex and Source of Hamster Affect Experimental Cholesterol Cholelithiasis

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In the present study, we examined the effect of the following factors on a hamster model of cholesterol cholelithiasis: (i) the source of the golden Syrian hamsters (Sasco, Omaha, NE or Charles River, Wilmington, MA), (ii) the sex of the experimental animals and (iii) their age (4 wk *vs.* 8 wk of age). All hamsters were fed a semipurified diet which contained cholesterol (0.3%) and palmitic acid (1.2%). No cholesterol gallstones formed in any of the female hamsters regardless of age or source. The 4-week-old male hamsters from Sasco had the greatest incidence of gallstones (93%). The 8-week-old male hamsters tended to have a lower incidence of cholesterol gallstones than the younger ones, regardless of the commercial supplier (67 *vs.* 93% for Sasco and 27 *vs.* 40% for Charles River). Female hamsters had higher liver and serum cholesterol levels than the male hamsters; Charles River hamsters had lower serum cholesterol concentrations than the Sasco animals. Total biliary lipid concentrations were highest in Sasco male hamsters, but biliary cholesterol (mol%) was lower in the males than in the females (4.2–4.5% *vs.* 6.1–7.1%) regardless of age. The cholesterol saturation indices were higher in the Sasco females than the corresponding males; these values were lower in the Sasco hamsters than the Charles River animals, regardless of age or sex. The male Sasco hamsters had a higher total biliary bile acid concentration (98.9 mg/mL) than the Sasco females (58.9 mg/mL) and the Charles River animals (24.6 mg/mL for males and 38.2 mg/mL for females). The percentage of chenodeoxycholic acid in bile was significantly lower, and the percentage of cholic acid was higher in all females as compared to males. We conclude that there is a sex, age and "strain" difference in cholesterol cholelithiasis in hamsters; it is important to consider these factors when working with the hamster model of gallstone disease. All female hamsters were markedly resistant to the induction of cholesterol gallstone disease.

Lipids 28, 981–986 (1993).

A number of hamster models has been used to study cholesterol cholelithiasis (1–5). In our laboratory, hamsters are fed a nutritionally adequate semipurified diet (SPD) containing cholesterol (0.3%) and butterfat (4.0%) to produce cholesterol gallstones in 50–60% of the animals (6). When palmitic acid, a major fatty acid component of butterfat, was substituted for butterfat, gallstone incidence increased to 90% or higher (7). These earlier studies used young (4-week-old) male golden Syrian hamsters from Sasco Inc. (Omaha, NE), since these animals gave the best and most reproducible incidence of cholesterol stones. Hamsters from other commercial suppliers (such as, Charles River Breeding Labs, Wilmington, MA) were significantly less susceptible to the development of gallstones (5,6).

We have now examined the effect of age (4-week-old *vs.* 8-week-old hamsters), sex (male *vs.* female animals) and commercial source of hamster (Sasco *vs.* Charles River) on the incidence of cholelithiasis. All animals were fed a lithogenic diet containing 0.3% cholesterol and 1.2% palmitic acid. Our results showed that male Sasco hamsters, 4 wk of age, had the greatest incidence of gallstones. Gallstone incidence tended to decrease in the older, 8-wk, animals. Interestingly, no cholesterol gallstones formed in any female hamsters, regardless of age or source.

MATERIALS AND METHODS

Animals. Male and female, golden Syrian hamsters (*Mesocricetus auratus*), 4-week-old (48–62 g) and 8-week-old (93–111 g), were obtained from two different suppliers, namely, Sasco and Charles River. The animals were maintained with water and Purina rodent chow *ad libitum* for a 1-wk quarantine period prior to the start of the experiment. All hamsters were fed the following pelleted, color-coded lithogenic diet (Dyets, Inc., Bethlehem, PA): 46.2% corn starch, 20.0% casein, 14.6% dyetrose (soluble starch), 10.0% fiber (cellulose), 5.0% salt mix (modified U.S.P. XIV salt mix, no. 200951), 2.0% corn oil, 1.2% palmitic acid, 0.5% vitamin mix (no. 300000), 0.3% cholesterol, 0.2% choline chloride. The hamsters were randomly divided into eight groups as follows: group 1, 4-week-old Sasco males; group 2, 4-week-old Sasco females; group 3, 4-week-old Charles River males; group 4, 4-week-old Charles River females; group 5, 8-week-old Sasco males; group 6, 8-week-old Sasco females; group 7, 8-week-old Charles River males; group 8, 8-week-old Charles River females. At the end of the 6-wk feeding period, the hamsters were fasted for 24 h and then anesthetized with 20 mg of ketamine hydrochloride (Aveco Co., Inc., Fort Dodge, IA). Blood was withdrawn by cardiac puncture for the determination of serum cholesterol. The gallbladder was examined for the presence of gallstones. Bile was removed with a syringe and examined under a polarizing light microscope (Olympus MCHAP microscope; Olympus Corp., Lake Success, NY) for the presence of cholesterol crystals or liquid crystals. The remaining bile was aliquoted for the determination of biliary lipids. The liver was excised and weighed, and portions were taken for cholesterol analysis. Liver segments from hamsters in the 4-week-old categories were fixed in Millonig's buffered formalin, then embedded in paraffin, sectioned and stained with hematoxylin and eosin. Using a grade scale of 0 to ++++, the pathologist

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; CSI, cholesterol saturation index (indices); DCA, deoxycholic acid; 7-keto-DCA, 3α,12α-dihydroxy-7-keto-5β-cholenic acid; FT-IR, Fourier transform infrared; SPD, semipurified diet.

evaluated the sections without knowledge of the treatment category of the hamsters. This procedure was similar to that used in a prior study (8).

Analytical procedures. Biliary bile acids were determined as the methyl ester acetates using a SPB-5 15 m capillary column (Supelco, Bellefonte, PA) attached to a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA); 3α, 7α-dihydroxy-12-keto-5β-cholanoic acid was used as an internal standard (8). Biliary phospholipids were determined by an enzymatic-colorimetric procedure using the Wako phospholipid B kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cholesterol concentrations of bile, liver and serum were determined by gas–liquid chromatography on a 0.5% OV-210 packed column, using 5α-cholestane as an internal standard as described previously (9).

Gallstones were analyzed by diffuse reflectance Fourier transform infrared (FT-IR) spectroscopy, using a Perkin-Elmer 1710 spectrometer (Perkin-Elmer Corp., Norwalk, CT) attached to a Perkin-Elmer 7500 laboratory computer (10).

Calculations and statistics. The cholesterol saturation indices (CSI) were determined using published methods (11,12). The data were reported as the mean ± SD. Differences between the various experimental groups were calculated using ANOVA to determine the F statistic; Student's *t*-test was applied to those values for which the F statistic was significant ($P < 0.05$) (13,14).

RESULTS

All hamsters were fed the experimental diets for a 6-wk period. The animals remained healthy throughout the experiment. The 4-week-old males, from either Sasco or Charles River, gained more weight than the corresponding female hamsters (Table 1); 44 *vs.* 32 g for Sasco and 50 *vs.* 34 g for Charles River. The 8-week-old hamsters maintained the same weight or gained less weight than the corresponding 4-week-old animals. There were no significant differences in food intake (10–11 g/d) for all eight groups. On the average, liver weights of female hamsters were larger than males for both Sasco (groups 2 and 6 *vs.* groups 1 and 5; 6.8 and 8.3 *vs.* 6.2 and 6.1 g) and Charles River (groups 4 and 8 *vs.* groups 3 and 7; 5.4 and 7.5 *vs.* 5.6 and 6.0 g).

In all hamsters from the 4-week-old categories, the liver sections revealed minimal (+) bile duct proliferation in the portal tracts. In the livers of the Sasco hamsters, both male and female, the bile duct proliferation was accompanied by a mild (+) infiltration of mononuclear cells, mostly small lymphocytes. This was conspicuously absent from the portal tracts of the Charles River animals. Microvesicular parenchymal steatosis was evident in all of the 4-week-old hamsters, generally graded as ++ to +++, except for the Sasco males wherein the steatosis was often minimal (+). The 4-week-old Sasco females also exhibited multiple fatty cysts, a phenomenon that was less prevalent or absent from the livers of hamsters in the other 4-week-old categories. Focal infiltrates of mononuclear cells in the lobular parenchyma were noted in all of the 4-week-old hamster livers, generally graded as +, except for the Sasco females which frequently displayed more prominent parenchymal infiltrates (++ to +++).

Gallstone incidence was determined at sacrifice (Table 2). No cholesterol gallstones formed in any of the female hamsters, regardless of age or source. Cholesterol cholelithiasis in the males showed marked variability. In all cases, the male Sasco hamsters had a greater incidence of cholesterol gallstones than the male Charles River hamsters: group 1 *vs.* group 3, 14/15 *vs.* 6/15 for 4-week-old, and group 5 *vs.* group 7, 10/15 *vs.* 4/15 for 8-week-old animals. The older hamsters tended to have a lower incidence of cholesterol gallstones than the younger animals. Some 8-week-old male Sasco hamsters had pigment stones in the gallbladder (group 5, 4/15). The pigment stones consisted mainly of calcium phosphate, protein and calcium bilirubinate. In the 4-week-old and 8-week-old Charles River animals, females had more pigment stones than the males. A few of the Sasco females had pigment stones but there were no significant differences among the groups.

Table 3 summarizes the cholesterol concentrations in liver, serum and bile. In all cases, regardless of source or age, female hamsters had higher liver cholesterol levels than males: groups 2 and 6 *vs.* groups 1 and 5; 64.30 and 60.52 *vs.* 34.99 and 43.68 mg/g in Sasco hamsters, and groups 4 and 8 *vs.* groups 3 and 7; 60.10 and 57.31 *vs.*

TABLE 1

Body Weight, Food Intake and Liver Weight of Sasco and Charles River Hamsters on the Palmitic Acid Diet[a]

Group	Source	Age/sex	Number of animals	Initital weight (g)	Final weight (g)	Food intake (g/d)	Liver weight (g)
1	Sasco	4 wk-M	15	61 ± 2	105 ± 8[b]	10 ± 1	6.2 ± 0.6[c]
2	Sasco	4 wk-F	13	62 ± 3	94 ± 7	11 ± 1	6.8 ± 0.9
3	Charles River	4 wk-M	15	53 ± 4	103 ± 12[d]	10 ± 1	5.6 ± 0.7
4	Charles River	4 wk-F	18	48 ± 3	82 ± 6	10 ± 0	5.4 ± 0.5
5	Sasco	8 wk-M	15	111 ± 4	110 ± 11	10 ± 1	6.1 ± 0.9[e]
6	Sasco	8 wk-F	16	107 ± 4	124 ± 6	11 ± 0	8.3 ± 0.6
7	Charles River	8 wk-M	15	93 ± 5	108 ± 9	10 ± 1	6.0 ± 0.9[f]
8	Charles River	8 wk-F	16	93 ± 4	104 ± 8	11 ± 0	7.5 ± 1.1

[a]See Materials and Methods for details of the hamster diet. The age refers to the age of the hamsters at the start of the experimental feeding. All hamsters were fed the diet for 6 wk. Numbers are mean ± SD. M, male; F, female. [b]Differs from group 2, $P < 0.01$. [c]Differs from group 2, $P < 0.05$. [d]Differs from group 4, $P < 0.01$. [e]Differs from group 6, $P < 0.01$. [f]Differs from group 8, $P < 0.01$.

TABLE 2

Gallstone Incidence in Sasco and Charles River Hamsters[a]

Group	Source	Age/sex	Cholesterol gallstones (%)	Pigment gallstones (%)
1	Sasco	4 wk-M	14/15 (93)[b]	0/15 (0)[c]
2	Sasco	4 wk-F	0/13 (0)	1/13 (8)
3	Charles River	4 wk-M	6/15 (40)[d]	0/15 (0)[e]
4	Charles River	4 wk-F	0/18 (0)	6/18 (33)
5	Sasco	8 wk-M	10/15 (67)[f]	4/15 (27)
6	Sasco	8 wk-F	0/16 (0)	1/16 (6)
7	Charles River	8 wk-M	4/15 (27)[g]	0/15 (0)
8	Charles River	8 wk-F	0/16 (0)	3/16 (19)

[a]See Materials and Methods for details of the hamster diet. M, male; F, female.
[b]Differs from groups 2-4 and 6-8, $P < 0.005$.
[c]Differs from group 4, $P < 0.025$; group 5, $P < 0.05$.
[d]Differs from group 4, $P < 0.005$.
[e]Differs from group 4, $P < 0.025$.
[f]Differs from group 6, $P < 0.005$; group 7, $P < 0.05$.
[g]Differs from group 8, $P < 0.05$.

42.02 and 44.51 mg/g in Charles River hamsters. Serum cholesterol in Sasco males (groups 1 and 5, 311 and 326 mg/dL) was lower than in the corresponding female hamsters (groups 2 and 6, 491 and 552 mg/dL) regardless of age. The male Charles River hamsters also had lower serum cholesterol (groups 3 and 7, 294 and 262 mg/dL) compared to the females (groups 4 and 8, 308 and 376 mg/dL); in all cases, Charles River hamsters had lower serum cholesterol than the Sasco animals. Biliary cholesterol was higher in 4-week-old male Sasco hamsters compared to the corresponding Charles River animals (group 1 *vs.* group 3, 4.46 *vs.* 2.36 mg/mL). Four-week-old Charles River males had lower biliary cholesterol levels than 8-week-old males (group 3 *vs.* group 7, 2.36 *vs.* 3.75 mg/mL). Interestingly, 8-week-old Charles River males had significantly higher bile cholesterol than the females (group 7 *vs.* group 8, 3.75 *vs.* 2.56 mg/mL).

Biliary lipids and cholesterol saturation indices are summarized in Table 4. In Sasco hamsters, the mol% biliary cholesterol was lowest in the males (groups 1 and 5, 4.2 and 4.5%) as compared to females (groups 2 and 6, 6.1 and 7.1%) regardless of age. In the Charles River groups, the mol% cholesterol was higher in the 8-week-old *vs.* the 4-week-old animals regardless of sex (groups 7 *vs.* 3, 9.5 *vs.* 7.8% and groups 8 *vs.* 4, 8.7 *vs.* 7.1%). Charles River males had a higher mol% phospholipid than the corresponding Sasco males (groups 3 and 7 *vs.* groups 1 and 5; 14.1 and 17.7 *vs.* 10.9 and 9.9%). The total lipids were higher in Sasco males than the corresponding females (groups 1 and 5 *vs.* groups 2 and 6; 14.9 and 14.6 *vs.* 9.4 and

TABLE 3

Cholesterol Levels in Sasco and Charles River Hamsters[a]

Group	Source	Age/sex	Liver (mg/g)	Serum (mg/dL)	Bile (mg/mL)
1	Sasco	4 wk-M	34.99 ± 10.42[b]	311 ± 43[b]	4.46 ± 1.44[c]
2	Sasco	4 wk-F	64.30 ± 6.90	491 ± 79[d]	4.20 ± 1.31
3	Charles River	4 wk-M	42.02 ± 11.86[e]	294 ± 42[f]	2.36 ± 1.14[g]
4	Charles River	4 wk-F	60.10 ± 12.57	308 ± 33[h]	3.34 ± 1.35
5	Sasco	8 wk-M	43.68 ± 6.37[i]	326 ± 27[j]	4.28 ± 1.15
6	Sasco	8 wk-F	60.52 ± 5.29	552 ± 66[h]	3.49 ± 1.21
7	Charles River	8 wk-M	44.51 ± 9.62[k]	262 ± 38[h]	3.75 ± 1.17[h]
8	Charles River	8 wk-F	57.31 ± 16.02	376 ± 67	2.56 ± 0.99

[a]See Materials and Methods for details of the hamster diet. Numbers are mean ± SD. M, male; F, female.
[b]Differs from group 2, $P < 0.01$. [c]Differs from group 3, $P < 0.01$. [d]Differs from group 4, $P < 0.01$; group 6, $P < 0.04$. [e]Differs from group 4, $P < 0.01$. [f]Differs from group 7, $P < 0.04$. [g]Differs from group 7, $P < 0.01$. [h]Differs from group 8, $P < 0.01$. [i]Differs from group 6, $P < 0.01$. [j]Differs from groups 6 and 7, $P < 0.01$. [k]Differs from group 8, $P < 0.02$.

TABLE 4

Effect of Age, Sex and Source on Biliary Lipids in the Hamster[a]

Group	Source	Age/sex	Cholesterol (mol%)	Phospholipids (mol%)	Bile acids (mol%)	Total lipid (g/dL)	Cholesterol saturation index
1	Sasco	4 wk-M	4.2 ± 1.3[b]	10.9 ± 2.2[c]	84.3 ± 4.1[d]	14.9 ± 7.1[e]	0.87 ± 0.25[b]
2	Sasco	4 wk-F	6.1 ± 1.2[f]	12.0 ± 3.2[g]	81.4 ± 4.7[g]	9.4 ± 3.6[h]	1.43 ± 0.43
3	Charles River	4 wk-M	7.8 ± 2.2[i]	14.1 ± 5.4[i]	76.9 ± 5.4[j]	4.1 ± 1.4[k]	1.67 ± 0.37
4	Charles River	4 wk-F	7.1 ± 1.1	15.1 ± 2.9[l]	77.5 ± 3.1	6.8 ± 2.8[l]	1.36 ± 0.29[l]
5	Sasco	8 wk-M	4.5 ± 1.8[m]	9.9 ± 2.9[n]	85.6 ± 4.3[n]	14.6 ± 4.4[m]	1.00 ± 0.36[m]
6	Sasco	8 wk-F	7.1 ± 0.8	10.9 ± 2.6	81.3 ± 2.4	6.6 ± 2.6[l]	1.82 ± 0.41[o]
7	Charles River	8 wk-M	9.5 ± 1.8	17.7 ± 1.8[l]	72.7 ± 2.3[l]	5.2 ± 1.2[o]	1.72 ± 0.20[l]
8	Charles River	8 wk-F	8.7 ± 2.1	10.9 ± 2.3	80.0 ± 3.8	3.9 ± 1.5	2.49 ± 0.91

[a]See Materials and Methods for details of the hamster diet. Numbers are mean ± SD. M, male; F, female. [b]Differs from groups 2 and 3, $P < 0.01$. [c]Differs from group 3, $P < 0.05$. [d]Differs from group 3, $P < 0.01$. [e]Differs from group 2, $P < 0.02$; group 3, $P < 0.01$. [f]Differs from group 4, $P < 0.03$; group 6, $P < 0.02$. [g]Differs from group 4, $P < 0.01$. [h]Differs from group 4, $P < 0.04$; group 6, $P < 0.03$. [i]Differs from group 7, $p < 0.03$. [j]Differs from group 7, $P < 0.02$. [k]Differs from group 4, $P < 0.01$; group 7, $P < 0.03$. [l]Differs from group 8, $P < 0.01$. [m]Differs from groups 6 and 7, $P < 0.01$. [n]Differs from group 7, $P < 0.01$. [o]Differs from group 8, $P < 0.02$.

TABLE 5

Biliary Bile Acid Composition in Sasco and Charles River Hamsters[a]

Group	Source	Age/sex	Total bile acid (mg/mL)	LCA (%)	DCA (%)	CDCA (%)	CA (%)	ACA (%)	HDCA (%)	MDCA (%)	7-keto-DCA (%)	Others (%)
1	Sasco	4 wk-M	98.9 ± 51.1[b]	5.2 ± 0.8[c]	12.6 ± 2.8[d]	38.4 ± 4.2[e]	23.4 ± 5.4[c]	0.9 ± 0.9[f]	12.0 ± 3.1[c]	3.4 ± 1.7[d]	0.3 ± 0.4[c]	2.6 ± 1.7
2	Sasco	4 wk-F	58.9 ± 22.7[g]	2.7 ± 2.2	14.3 ± 4.3	22.6 ± 5.6	42.9 ± 6.2	0.5 ± 0.4	4.2 ± 1.9[h]	2.8 ± 1.3[h]	2.9 ± 1.9	4.7 ± 0.9
3	Charles River	4 wk-M	24.6 ± 8.7[h]	1.2 ± 0.4[h]	5.3 ± 2.1[h]	40.8 ± 8.9[h]	34.7 ± 7.2[h]	2.4 ± 1.9[i]	0.6 ± 0.4	0.2 ± 0.3	6.6 ± 2.3[j]	6.8 ± 2.9
4	Charles River	4 wk-F	38.2 ± 15.9[k]	2.0 ± 0.9[k]	12.7 ± 1.9[j]	27.6 ± 4.8	46.7 ± 5.7	0.3 ± 0.5	0.4 ± 0.4	0.1 ± 0.2	4.4 ± 2.3[k]	5.2 ± 2.2
5	Sasco	8 wk-M	97.0 ± 30.6[m]	5.0 ± 0.8[m]	13.0 ± 3.9[n]	36.0 ± 6.4[m]	20.7 ± 5.5[m]	1.1 ± 0.7[m]	13.2 ± 2.5[m]	3.3 ± 1.0[n]	0.5 ± 0.4[m]	5.4 ± 0.9
6	Sasco	8 wk-F	41.0 ± 15.3[k]	2.3 ± 0.9[k]	14.5 ± 2.3[k]	21.3 ± 6.0[o]	44.9 ± 5.9	0.1 ± 0.2	5.3 ± 1.4[k]	2.9 ± 0.9[k]	2.8 ± 1.9[k]	4.9 ± 1.6
7	Charles River	8 wk-M	30.0 ± 8.2	1.5 ± 0.4[k]	5.4 ± 1.2[k]	43.3 ± 2.9[k]	34.4 ± 3.0[k]	4.1 ± 2.3[k]	0.5 ± 0.3	0.3 ± 0.3	4.8 ± 2.1[k]	4.7 ± 0.7
8	Charles River	8 wk-F	24.4 ± 9.4	1.0 ± 0.5	10.8 ± 3.1	25.7 ± 5.4	46.5 ± 6.2	0.2 ± 0.3	0.5 ± 0.4	0.2 ± 0.3	8.3 ± 4.2	6.2 ± 2.0

[a]See Materials and Methods for details of the hamster diet. Numbers are mean ± SD. M, male; F, female. LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; ACA, allocholic acid; HDCA, hyodeoxycholic acid; MDCA, murideoxycholic acid; 7-keto-DCA, 3α,12α-dihydroxy-7-keto-5β-cholanoic acid. [b]Differs from group 2, $P < 0.02$; group 3, $P < 0.01$. [c]Differs from groups 2 and 3, $P < 0.01$. [d]Differs from group 3, $P < 0.01$. [e]Differs from group 2, $P < 0.01$. [f]Differs from group 3, $P < 0.02$. [g]Differs from group 4, $P < 0.01$; group 6, $P < 0.02$. [h]Differs from group 4, $P < 0.01$. [i]Differs from group 4, $P < 0.01$; group 7, $P < 0.04$. [j]Differs from group 4, $P < 0.02$; group 7, $P < 0.04$. [k]Differs from group 8, $P < 0.01$. [l]Differs from groups 6 and 7, $P < 0.01$. [m]Differs from group 8, $P < 0.05$. [n]Differs from group 7, $P < 0.01$. [o]Differs from group 8, $P < 0.04$.

6.6 g/dL). On the average, the Sasco males had higher total lipids (groups 1 and 5, 14.9 and 14.6 g/dL) than the corresponding Charles River males (groups 3 and 7, 4.1 and 5.2 g/dL); a similar result was observed for the female hamsters (groups 2 and 6 *vs.* groups 4 and 8; 9.4 and 6.6 *vs.* 6.8 and 3.9 g/dL). The CSI were higher in the Sasco females than the males (groups 2 and 6 *vs.* groups 1 and 5; 1.43 and 1.82 *vs.* 0.87 and 1.00). On the average, the Sasco males had lower CSI than the Charles River males (groups 1 and 5 *vs.* groups 3 and 7; 0.87 and 1.00 *vs.* 1.67 and 1.72); similar results were found in female hamsters (groups 2 and 6 *vs.* groups 4 and 8; 1.43 and 1.82 *vs.* 1.36 and 2.49).

Table 5 summarizes the biliary bile acid composition in the gallbladder at the time of sacrifice. Male Sasco hamsters of both ages had a higher total bile acid concentration as compared to the corresponding females (groups 1 and 5 *vs.* groups 2 and 6; 98.9 and 97.0 *vs.* 58.9 and 41.0 mg/mL). The total bile acid concentration did not differ significantly within all four groups of Charles River hamsters. Overall, Charles River animals had lower total bile acid concentrations compared to Sasco hamsters: groups 3 and 7 *vs.* groups 1 and 5; 24.6 and 30.0 *vs.* 98.9 and 97.0 mg/mL for males, and groups 4 and 8 *vs.* groups 2 and 6; 38.2 and 24.4 *vs.* 58.9 and 41.0 mg/mL for females. The female Charles River hamsters tended to have a higher percentage of deoxycholic acid (DCA) than the males (groups 4 and 8 *vs.* groups 3 and 7; 12.7 and 10.8 *vs.* 5.3 and 5.4%). Chenodeoxycholic acid (CDCA) was significantly lower in all Sasco females compared to the males (groups 2 and 6 *vs.* groups 1 and 5; 22.6 and 21.3 *vs.* 38.4 and 36.0%). Similar results were observed for the Charles River hamsters: groups 4 and 8, 27.6 and 25.7% for females *vs.* groups 3 and 7, 40.8 and 43.3% for males. The percentage of cholic acid (CA) was higher for females from either Sasco or Charles River: for Sasco animals, groups 2 and 6 *vs.* groups 1 and 5, 42.9 and 44.9 *vs.* 23.4 and 20.7%; for Charles River animals, groups 4 and 8 *vs.* groups 3 and 7, 46.7 and 46.5 *vs.* 34.7 and 34.4% for females *vs.* males, respectively. The keto bile acid, 3α,12α-dihydroxy-7-keto-5β-cholanoic acid (7-keto-DCA acid), was present in smaller amounts in male Sasco hamsters (groups 1 and 5) 0.3 and 0.5% as compared to the females (groups 2 and 6) 2.9 and 2.8%. The proportion of this bile acid was higher in the Charles River hamsters: groups 3 and 7, 6.6 and 4.8% in males, and groups 4 and 8, 4.4 and 8.3% in females, than in the Sasco hamsters.

DISCUSSION

Attempts to induce cholesterol cholelithiasis in hamsters have given variable results. These differences can be attributed to changes in dietary components (6,15–17) and the source of the hamsters (5,6,18). Our laboratory has spent the last decade studying hamster models of cholelithiasis in an attempt to make them reliable and reproducible. We have now studied the effect of age, sex and source of hamsters on gallstone incidence.

Male and female hamsters, 4-week-old and 8-week-old from Sasco or Charles River, were fed nutritionally adequate SPDs containing 0.3% cholesterol and 1.2% palmitic acid for a 6-wk period. We have previously reported that the substitution of palmitic acid for butterfat in a lithogenic diet enhanced cholesterol gallstone

incidence in the 4-week-old male golden Syrian hamster from Sasco (7). The 8-week-old animals had initial weights similar to the final weights of the 4-week-old animals (groups 1–4). In the present study, palmitic acid did not enhance cholelithiasis in female hamsters as it did in males; no cholesterol gallstones formed in any of the females. Male Sasco hamsters had the greatest incidence of stones. In general, the cholelithiasis was more severe in young (4-week-old) hamsters than in the older (8-week-old) animals. The opposite effect has been found in humans, where gallstone incidence is more prevalent in females with increasing age (19). Dam (16), who studied hamsters many years ago, had shown that young hamsters tended to have more cholesterol gallstones than older animals. In addition, male hamsters tended to have a higher incidence of cholesterol stones than females. However, under the conditions employed, females, too, formed cholesterol gallstones (16). This is different from our results with female hamsters who had no stones. It should be noted that Dam's hamsters were of a different source, frequently became ill and died prematurely on the fatty acid-deficient diet. Hikasa *et al.* (20) also demonstrated the influence of age on the production of gallstones in the hamster, with the younger hamsters having a higher incidence. Trautwein *et al.* (21) recently published a comparison of gallstone incidence in three strains of hamsters: Charles River, Biobreeder (Ottawa, Canada) and Harlan Sprague-Dawley (Indianapolis, IN). Their study was not strictly comparable to the present experiments, since they used a "Dam-type" diet which contained 5% butter as fat, but very little essential fatty acids (21). A strain difference for gallstone incidence was observed, but the number, 5–7, of animals used was too small for statistical comparison.

Although female hamsters did not form cholesterol stones, they had high CSI. Cholesterol saturation of bile is a necessary, but obviously not the only, determinant of cholesterol stone formation (22). Our previous study and those of others have shown that there is an increase in cholesterol saturation of bile with advancing age in both sexes (23). However, total biliary lipid concentrations were significantly lower in female hamsters than in males. Studies in humans showed that nucleation times were prolonged in dilute gallbladder bile despite a very high CSI; concentrated bile had short nucleation times despite a relatively low CSI (24,25). In addition, individuals with a supersaturated bile may not form stones because the time required for cholesterol to nucleate may be longer than the residence time of the bile in the gallbladder (26). In our experiment, since female hamsters from both sources had more dilute bile than males, the nucleation time might have been prolonged, and gallstones did not form.

Cholesterol is solubilized in bile in two different forms: (i) vesicles composed mainly of cholesterol and phospholipid and (ii) micelles composed of cholesterol, phospholipid and bile salt (27–29). Cholesterol is known to nucleate from the vesicles; vesicles with a high cholesterol/phospholipid ratio are more prone to nucleation (30–33). Dilute bile may have a smaller amount of cholesterol in the vesicular form and therefore does not nucleate. In our experiments, the male Sasco hamsters have a twofold higher bile acid concentration in the gallbladder than the female hamsters from Sasco or the Charles River hamsters of either sex. Hamsters from Charles River have very dilute bile; the males have even more dilute bile than the females from Sasco, yet a small percentage of these hamsters formed gallstones. There must be other factor(s) besides biliary total lipid concentration that make female hamsters resistant to cholesterol gallstone formation; a hormonal effect should be considered. It is also reported that bile contains nucleating or antinucleating agents, presumably proteins, that play a role in cholesterol nucleation or lack thereof (34–37).

It has been shown that administration of estrogens to animals and humans decreased bile acid secretion, which would result in a rise in biliary cholesterol saturation (38–47). Estrogen prevents the stimulation of cholesterol 7α-hydroxylase by dietary cholesterol in the hamster (38,39,41). This may explain the lower bile acid concentration observed in female hamsters. A lower bile salt concentration reduces the number of micelles. It has been observed that as the number of micelles decreases, the cholesterol/phospholipid ratio of vesicles decreases, and nucleation time increases (48).

Biliary bile acid composition was markedly different among the various groups. The female hamsters had a lower CDCA concentration than the males. Hormones could be responsible for this effect, since the administration of estrogen has been found to reduce CDCA in bile (44,49). Sasco males had a higher percentage of hyodeoxycholic acid and murideoxycholic acid, but a lower percentage of 7-keto-DCA than the Sasco females or the Charles River hamsters of both sexes. These three bile acids are bacterial in origin. The formation of cholesterol gallstones could conceivably be affected by bacterial alteration of the biliary bile acid composition as some earlier investigators have indicated (50–52). The liver normally biotransforms ketonic secondary bile acids, such as 7-keto-DCA to hydroxy acids (*e.g.*, CA) by reduction of the keto group. Keto bile acids are more common in patients with liver disease (53). Female hamsters may conceivably have some liver injury as a result of the cholesterol diet, but a possible role of the 7-keto bile acid, in particular, and bile acid composition, in general, in lithogenesis remains unknown.

Female hamsters had larger livers and higher liver cholesterol levels than the males, regardless of age or source. Females store more cholesterol esters in the liver than males (54). The accumulation of the cholesterol ester in the liver is associated with suppression of cholesterol synthesis (54). This difference between male and female hamsters might also be hormonal in origin. Coyne *et al.* (38) reported that estrogen-treated hamsters on a high cholesterol diet had increased liver weights, which may be explained in part by an impaired ability to excrete the high exogenous cholesterol load in the form of bile acids. The fatty cysts and parenchymal infiltrates in the 4-week-old Sasco females may reflect this impairment; however, similar changes were either less prevalent or absent from the livers of hamsters in the other 4-week-old categories, including the Charles River females. While none of the observed morphologic changes should be construed as reflecting substantial or irreversible liver damage, still it is useful to know that the same diet can elicit different morphologic alterations in the livers of hamsters, depending on sex and source.

Our studies show that the "best" model for gallstone formation is the 4-week-old Sasco male hamster fed

palmitic acid. Although our animal model is not identical to human cholelithiasis, it should enable us to systematically examine how certain dietary components (fat, fiber), hormones and synthetic cholelitholytic agents affect stone incidence. In addition, because experiments (stone formation) can be completed in only 6 wk, and bile can easily be obtained, this model is clearly superior to studies in humans.

In conclusion, sex, age and source are important when working with the hamster model of cholesterol cholelithiasis. Our results show for the first time that age and sex may affect cholesterol cholelithiasis in hamsters fed nutritionally adequate diets.

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Preferential Reduction in Adipose Tissue α-Linolenic Acid (18:3ω3) During Very Low Calorie Dieting Despite Supplementation with 18:3ω3[1]

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We have previously reported that the relative content of 18:3ω3 in adipose triglyceride (TG) of women was reduced following major weight loss while on a very low calorie diet (VLCD). In an attempt to prevent this loss of 18:3ω3 reserves, we have tested two VLCD supplemented with varying amounts of 18:3ω3. The formula (FORM) and food VLCD (2.1–3.0 MJ or 500–700 kcal/d) contained 20 g/d of fat and provided the recommended dietary allowance for minerals and vitamins. FORM subjects (Group 1) were 5 women [initial body mass index (BMI) of 36.8, 168% ideal body weight (IBW) who received 20 g/d of canola oil (1.6 g 18:3ω3). Their mean weight loss was 23.9 kg in a 4–5 mon period. Food VLCD subjects (Group 2) were 6 women (BMI 33.9, 155% IBW) supplemented with 2 g/d of linseed oil (1.1 g 18:3ω3). Their mean weight loss was 17.4 kg in a 2–3 mon period. Needle biopsies of adipose tissue were obtained from Group 1 before, at midpoint and after weight loss; and from Group 2 before and after weight loss. The adipose TG and serum (Group 1) were separated and their fatty acid composition determined by thin-layer and gas chromatography. In Group 1, adipose 18:3ω3 fell from 0.65 to 0.59 wt%, then to 0.52 wt% during weight loss. In Group 2, it fell from 0.77 to 0.64 wt%. The fall in adipose 18:3ω3 with weight loss was significant at $P = 0.01$ (Group 1) and $P < 0.01$ (Group 2). There were no differences between responses to the 1.1 g/d or 1.6 g/d 18:3ω3 supplements. The relative content of 18:3ω3 in serum free fatty acids from Group 1 was reduced after major weight loss. Thus, in both groups the ω3 supplementation did not help to maintain adipose tissue 18:3ω3 during rapid weight loss, and its decrement may affect circulating lipid pools. As adipose 18:2ω6 did not change with weight loss, this reduction in the ratio of ω6 precursor to ω3 precursor could eventually alter the balance of their products as well.
Lipids 28, 987-993 (1993).

Very low calorie diets (VLCD; less than 3.3 MJ/d or 800 kcal/d) have been used extensively to achieve rapid weight loss. Many concerns regarding the safety of VLCD in treating moderate to severe obesity have been addressed in the past two decades (1,2), but the issue concerning the degree to which functional tissue is preserved during and after major weight loss remains. Thus, attention has been focused on nutrient composition, especially high quality proteins and supplements, and their relation to preserving lean tissue and vital organ function (3–6). However, limited studies have been done to examine the effects of VLCD with varying fat composition on adipose essential fatty acid (EFA) reserves and their content in serum lipid fractions (7,8).

Humans undergoing rapid weight loss during a VLCD derive a majority of their daily energy needs from adipose tissue. In addition to the oxidation of fatty acids from adipose triglycerides (TG) to meet energy needs, EFA are mobilized to meet other physiological and metabolic needs. The effect of weight loss on adipose tissue composition has received little attention since the pioneering work of Hirsch (9) over 30 years ago. Given a 2.5 MJ (600 kcal) per day fat-free diet for up to 150 d, Hirsch (9) reported that obese humans showed no change in the proportion of palmitic (16:0), oleic (18:1ω9) and linoleic acids (18:2ω6) in adipose tissue, despite over 30 kg of weight loss. The response of the lesser components, such as arachidonic acid (20:4ω6), α-linolenic acid (18:3ω3), eicosapentaenoic acid (20:5ω3; EPA) and docosahexaenoic acid (22:6ω3; DHA), to weight loss was not reported in those studies. However, the fate of these minor components of adipose tissue are of increasing interest in view of their important roles in membrane function, where they do exist in greater concentration, and from which substrates for eicosanoid synthesis are derived. Furthermore, the relative homogeneity of adipose tissue composition among people of diverse ethnic backgrounds and diets (10–12) suggests that its composition is regulated to some degree, possibly to fulfill some metabolic or physiological need.

In a previous study (13) we examined adipose fatty acid composition of two groups of obese women on VLCD before and after undergoing 15–30 kg of weight loss. Neither diet group received ω3 fatty acid supplements, and the daily intake of this class of fatty acid was estimated at <200 mg. Following weight loss, their remaining body adipose tissue content of 18:1ω9, 18:2ω6 and 20:4ω6 had not changed, but 18:3ω3 fell significantly in the first group (0.67 to 0.56 wt%, $P < 0.001$) and similarly in the second (0.71 to 0.59 wt%, $P = 0.03$).

α-Linolenic acid cannot be synthesized endogenously by humans and must be obtained through their diet. It is also the metabolic precursor for the elongation and desaturation products 20:5ω3 and 22:6ω3. Dietary intake of 20:5ω3 and 22:6ω3 has been associated with reduced risk for atherosclerosis (14–16). Repeated weight loss in adults has been found to predispose them to early atherosclerosis (17,18), perhaps due to the depletion of ω3 fatty acid stores. Although the essentiality of the ω3 family remains a topic of debate, the two case reports in the literature of symptomatic human deficiency (19,20) suggest that the minimum maintenance ω3 fatty acid intake should be 0.5–1.0% of calories. A recent study of young adults reported the intake of 18:3ω3 at 0.3% of total energy (21). This implies that the ω3 nutriture of much of the population may be marginal. Seen from

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Abbreviations: BMI, body mass index (weight in kilogram divided by the square of height in meters); DHA, docosahexaenoic acid (22:6ω3); EPA, eicosapentaenoic acid (20:5ω3); EFA, essential fatty acid; FFM, fat-free mass; GC, gas chromatography; IBW, ideal body weight; PL, phospholipids; RDA, recommended dietary allowance; TG, triglyceride; TLC, thin-layer chromatography; VLCD, very low calorie diet(s).

this perspective, the loss of 18:3ω3 from adipose TG during rapid weight loss may have important health implications.

Due to our previous findings (13) and the evidence discussed now on the importance of ω3 fatty acids in metabolism, we have conducted a follow-up study. We have assessed whether additional supplementation of VLCD with 1.1 or 1.6 g/d of 18:3ω3 helps to maintain 18:3ω3 levels in adipose and serum during prolonged dieting (for up to six months) in obese females.

MATERIALS AND METHODS

This project was conducted with two separate groups of subjects. Both groups were studied in a multidisciplinary weight management clinic at the University of California at Davis. The subjects received one of two VLCD providing between 1.9–3.0 MJ (450–700 kcal) daily with different protein, carbohydrate and fat contents. The study protocols were approved by the Human Subjects Review Committee at the University of California at Davis. Written informed consent was obtained from each subject prior to participation.

Subjects. Volunteers for this study were recruited from a group of subjects who responded to newspaper advertisements. Preselection criteria included normal medical history, physical examination, electrocardiogram and routine hematological and serum biochemical studies to rule out cardiac, hepatic, renal, thyroid and pancreatic beta-cell dysfunction. All subjects were weight stable within a 3 kg range for the month prior to the study. An initial diet history indicated no restriction of animal products or vegetable fats in their habitual diets. Group 1 contained 5 obese females ages 38 to 44. They were from 25 to 76 kg above ideal body weight [IBW; taken as the median value of the medium frame range from the Metropolitan Life Insurance Tables (22)]. The subjects were given a formula VLCD containing canola oil. Group 2 contained 6 obese females ages 37 to 44. They were from 15 to 64 kg above their IBW. These subjects were assigned to a common food VLCD and supplemented with linseed oil capsules. The characteristics of Group 1 and Group 2 subjects are shown in the Results section in Table 1.

Diets. During the first two weeks of the outpatient protocol, all subjects were prescribed a 4.1 MJ/d (1000 kcal/d diet) common food diet containing 90 g of protein. Following the two-week adaptation period, subjects were assigned to one of two VLCD.

The formula diet (Sandoz, Minneapolis, MN) provided a daily intake of 2.8 MJ (660 kcal) with 90 g protein, 30 g carbohydrate and 20 g of fat. The protein source for this formula was pasteurized egg albumin and casein, and the fat source was canola oil (11% saturated, 58% monounsaturated, 23% 18:2ω6 and 8% 18:3ω3). The mineral and vitamin content of the formula met or exceeded the recommended dietary allowances (RDA) (23) for minerals, trace minerals and vitamins. Subjects in both groups were also prescribed 1.5–2.0 L of noncaloric fluids and up to 3 g of sodium daily with bouillon to maintain euvolemia despite the nutritional ketosis induced by the VLCD.

The common food VLCD consisted of three daily portions of lean meat, low-fat fish or poultry providing less than 10 g of carbohydrate and 1.5 g protein per kg IBW in 1.9–2.5 MJ (450–600 kcal) daily. The lipid content of this diet consisted of those fats inherent in the foods selected, and was judged by diet history to range from 15–20 g/d. Based upon the diet histories of the subjects and published tables for the fatty acid contents of these animal fats, approximately 10% of this dietary fat was 18:2ω6 and 1% 18:3ω3 (for estimated daily intakes of 2 g and 0.2 g, respectively). The mean ω3 fatty acid intake from fish as 20:5ω3 and 22:6ω3 was estimated at less than 100 mg/d. This diet was supplemented daily with 2 linseed oil capsules (1.1 g/d 18:3ω3), 25 mEq of potassium bicarbonate (K-Lyte, Bristol, Evansville, IN), 4 tablets of calcium/magnesium antacid (Calcitrel, Sterling, New York) providing 800 mg and 200 mg of these minerals, respectively, and a multivitamin with minerals (Centrum, Lederle). In total, the food portions plus supplements provided at least 100% of the RDA (23) for the major and trace minerals and vitamins. The calories given to all subjects on the formula diet were fixed; whereas the common food VLCD intake was variable, depending upon stature. The range of energy intakes between groups during the VLCD varied from 660 kcal/d for the former to 450–600 kcal/d for the latter. Thus, the diets were similar in energy content but were neither precisely isocaloric nor isonitrogenous. Their total ω3 contents from all sources were 1.6 g/d and 1.4 g/d, respectively.

Monitoring. Body weight and breath acetone (by gas chromatography; Caldetect Inc., Richmond, CA) were measured weekly. The weekly weights and breath ketones effectively monitored for subject adherence to the diets during the outpatient protocol.

Body composition analysis. Body composition of Group 1 was determined by hydrostatic weighing, as previously published (13), before dieting, at approximately 12–15 kg of weight loss, and following 17–30 kg of weight loss; while body composition of Group 2 was done before dieting and following 7–26 kg of weight loss. Body density and percent body fat were calculated using the Siri formula (24) and Brozek equation (25), respectively. Fat-free mass (FFM) was calculated as body weight minus total body fat.

Serum sampling and analysis. Fasting blood was drawn by venipuncture with minimal hemostasis from Group 1 subjects before the initial diet, early in the diet (month 2) and late in the diet (month 4 to month 6). Lipids were extracted from 2 mL of serum using 6 mL of chloroform/methanol (2:1, vol/vol) (26). The phospholipid, free fatty acid, TG and cholesteryl ester fractions were separated by thin-layer chromatography (TLC), transesterified using 5% methanolic HCl, and their fatty acid composition was determined by capillary gas chromatography (GC) (13,27). The serum cholesteryl ester data are not reported, due to difficulties with sample recovery following the TLC step.

Adipose tissue sampling and analysis. Subcutaneous adipose tissue was obtained by biopsy with a 14-gauge needle following local anesthesia. Group 1 subjects were biopsied from the lateral thigh before and after a mean of 14 kg of weight loss, and after a mean of 24 kg lost. The initial and final serum samples coincided with the initial and final adipose biopsies. Group 2 subjects were biopsied from the lateral thigh before and after a mean of 17 kg weight loss. After careful washing in saline solution and blotting, the adipose tissue was extracted in a ground glass homogenizer (26), the triglyceride fraction

TABLE 1

Characteristics of Subjects

	Age (yr)	Height (cm)	Initial weight (kg)	%IBW[a]	BMI[b]	Weight loss (kg)	Weight loss rate (kg/wk)
Group 1 (formula VLCD[c] containing canola oil)							
Mean	41	168	104.6	169	36.8	23.9	1.21
SEM	1	4	11.0	11	2.5	2.4	0.10
Group 2 (food VLCD supplemented with linseed oil)							
Mean	39	166	92.1	154	33.9	17.4	1.51
SEM	2	2	7.1	15	3.2	3.0	0.17

[a]IBW, ideal body weight, taken at mid-range of medium frame value.
[b]BMI, body mass index (weight in kilogram divided by the square of the height in milligrams).
[c]Very low calorie diet(s).

was separated by TLC and its fatty acid composition was determined by capillary GC. The specific details of this procedure have recently been published in detail (13).

Statistical analysis. Data analysis was performed using the PC-SAS (28) package on a microcomputer. Changes in the level of each specific fatty acid were assessed in Group 1 by repeated measures analysis of variance (ANOVA), and in Group 2 by paired *t*-test. Due to the number of individual tests done with each diet group, the threshold for significance was $P < 0.01$. Data are reported as means (SEM).

RESULTS

The characteristics of the two groups of subjects participating in this study are shown in Table 1. The two groups had similar age, height, initial body weight, %IBW, and body mass index (BMI). All the subjects exceeded the standards for obesity as determined by percent IBW and BMI, and all lost at least 0.9 kg per week. The mean rates of weight loss for the Group 1 and 2 subjects are shown in Table 1, and each subject's cumulative loss in Figure 1.

The data for Figure 1 are calculated as percent loss from initial weight, and this figure illustrates the consistent rate of loss within and between diet groups. These results indicate that little, if any, additional fat calories were consumed during the VLCD by our subjects. Adherence to the VLCD was further supported by breath acetone determinations. The subjects achieved and sustained values exceeding the equivalent of 0.4 mM β-hydroxybutyrate during the VLCD. This increase over baseline values (less than 0.05 mM in the pre-weight loss phase) indicated consistent avoidance of unprescribed dietary carbohydrate.

Body composition data for Group 1 and Group 2 are shown in Table 2. Total adipose weights are provided at each time point, plus total change for these parameters and FFM across weight loss. The changes in body composition were similar for both diets. There were no significant differences between the diets for total weight loss, fat loss or loss of FFM. A mean weight loss of 23.9 ± 2.4 kg was observed in the Group 1 and 17.4 ± 3.0 kg in the Group 2 subjects, corresponding to 22.8 and 18.2% reduction in initial body weight. The weight loss as fat was 87% for Group 1 and 70% for Group 2 subjects.

The fatty acid contents of adipose tissue from the lateral thigh regions of Group 1 and Group 2 subjects are shown in Table 3. For Group 1, α-linolenic acid declined significantly with weight loss from 0.65 to 0.52 wt% (20%, $P = 0.01$). Using the body composition data, the loss of 18:3ω3 from adipose stores was 1.2 g/d. Oleic acid and 22:5ω3 both showed slight rises after weight loss ($P = 0.007$ and $P = 0.003$, respectively), and 18:3ω6 showed a slight decline ($P = 0.006$). For Group 2, the only significant change with weight loss occurred in 18:3ω3, which fell from 0.77 to 0.64 wt% (17%, $P < 0.01$). Again from the body composition data, the rate of 18:3ω3 loss from adipose stores was 1.6 g/d.

The fatty acid constituents of the serum phospholipid (PL) fraction of Group 1 at three time points before and during the rapid weight loss phase are shown in Table 4. There were no significant changes in the PL ω6 or ω3 fatty acids. The serum free fatty acids for Group 1 subjects are shown in Table 5. Among the nonessential fatty acids, 14:0 declined ($P = 0.003$) with the VLCD, and 18:1ω9 rose ($P = 0.005$). Among the ω6 family, the only change was a fall in 18:3ω6 ($P = 0.01$). In the ω3 class, 18:3ω3 sustained a significant ($P < 0.01$) decline. Neither 20:5ω3 nor 22:6ω3 underwent significant change. The serum TGs for the Group 1 subjects are shown in Table 6. There were no

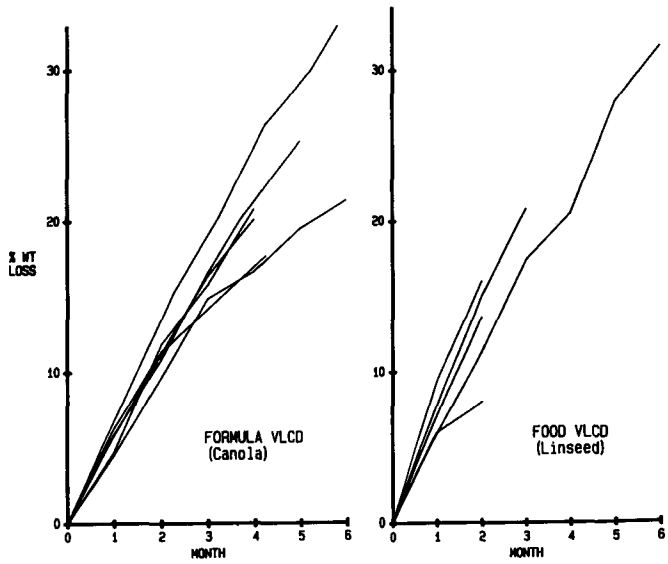

FIG. 1. Rates of weight loss for formula (Group 1) and common food very low calorie diets) (VLCD) (Group 2) subjects, calculated as percent of initial weight, and indicating uniform adherence to the VLCD.

TABLE 2

Body Composition[a]

Subject	Before		During		After		Change with VLCD		
	Total	Adipose	Total	Adipose	Total	Adipose	Total	FFM	Adipose
Group 1									
Mean	104.6	50.6	90.2	42.8	80.7	29.9	23.9	3.2	20.7
SEM	11.0	6.1	10.6	4.6	9.6	5.3	2.4	1.2	1.7
Group 2									
Mean	92.1	40.0	n.d.	n.d.	74.8	27.8	17.4	5.2	12.2
SEM	7.1	5.2	n.d.	n.d.	4.4	3.5	3.0	1.5	2.0

[a]Total weight, adipose weight and fat-free mass (FFM) in kg; n.d., not determined. VLCD, very low calorie diet(s).

significant changes among the nonessential fatty acids. In the ω6 family, 18:3ω6 fell ($P < 0.01$ for both). There were no significant changes in the ω3 family with the VLCD.

DISCUSSION

Our previous study had shown that VLCD employed for rapid weight loss in humans selectively reduce the content of α-linolenic acid in the remaining body adipose pool (13). This finding was consistent across two separate groups of subjects experiencing 15–25 kg of weight loss and was independent of the type of VLCD (food or formula) employed. It also implied a need for ω3 fatty acid supplementation to avoid tissue reduction of this nutrient. In the present study, we assessed two VLCD providing 1.6 g/d of 18:3ω3 (formula diet containing canola oil) or 1.4 g/d (common food plus linseed oil capsules). The diet-induced reduction of 18:3ω3 in adipose TG was similar in the two groups and was comparable to that found in the previous study (in which the diets provided < 0.2 g ω3 daily). There were no other consistent changes in the adipose fatty acid composition in either diet group. Thus, the 18:3ω3 supplementation did not prevent the loss of 18:3ω3 from adipose stores during the VLCD.

The 18:3ω3 supplement dose used in this study was determined using the data from the body composition studies of our previous work (13). Assuming uniform loss from different anatomic sites, total α-linolenic acid disappearance from adipose TG was determined to be 171 g in 140 d, or 1.2 g/d. This amount represented about 0.5% of daily energy expenditure and is very close to the estimates of minimum human need determined in two case studies describing overt deficiency of this required nutrient (19,20). We decided on supplement doses for the present study that would equal (linseed) or exceed (canola) the projected minimum need, hoping that this would protect the relative content of 18:3ω3 in adipose triglyceride. Despite the supplements, however, the calculated rates of 18:3ω3 loss from adipose triglyceride were 1.2 and 1.6 g/d for Groups 1 and 2, respectively. Clearly these 18:3ω3 supplements did not protect adipose reserves. Linoleic acid mobilization, on the other hand, was determined to be 22 g/d, providing more than 10% of total energy needs. This

TABLE 3

Adipose Triglyceride Fatty Acid Composition

Fatty acids	Group 1 (wt%; n = 5)						Group 2 (wt%; n = 6)			
	Before		During		After		Before		After	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	2.41	0.07	2.36	0.05	2.27	0.07	2.59	0.15	2.33	0.07
16:0	19.07	0.32	19.60	0.20	19.27	0.21	18.87	0.47	22.20	3.29
18:0	2.82	0.23	2.96	0.19	2.88	0.16	2.47	0.21	2.70	0.24
16:1[a]	7.52	0.16	6.54	0.29	6.23	0.32	7.45	0.61	7.29	0.74
18:1[b]	45.27[d]	1.52	45.85	0.22	46.69[d]	0.26	44.17	0.38	43.01	2.41
20:1[c]	0.60	0.01	0.63	0.01	0.69	0.01	0.60	0.02	0.57	0.07
18:2ω6	15.60	0.51	15.43	0.45	15.41	0.42	16.55	0.83	15.36	1.57
18:3ω6	0.09[d]	0.00	0.08	0.00	0.07[d]	0.00	0.11	0.01	0.10	0.00
20:2ω6	0.22	0.00	0.23	0.01	0.26	0.01	0.23	0.01	0.22	0.02
20:3ω6	0.28	0.02	0.31	0.02	0.35	0.03	0.31	0.03	0.33	0.05
20:4ω6	0.40	0.01	0.42	0.02	0.41	0.02	0.46	0.06	0.45	0.07
22:4ω6	0.16	0.01	0.18	0.01	0.21	0.01	0.17	0.02	0.14	0.02
18:3ω3	0.65[d]	0.02	0.59	0.02	0.52[d]	0.01	0.77[e]	0.05	0.64[e]	0.05
20:5ω3	0.04	0.00	0.03	0.00	0.03	0.00	0.06	0.01	0.06	0.03
22:5ω3	0.15	0.01	0.15[d]	0.00	0.17[d]	0.01	0.14	0.02	0.20	0.04
22:6ω3	0.10	0.01	0.10	0.00	0.10	0.01	0.09	0.01	0.15	0.06

[a]Sum of 16:1ω9 and 16:1ω7.
[b]Sum of 18:1ω9 and 18:1ω7.
[c]Sum of 20:1ω9 and 20:1ω7.
[d]Values differ $P \leq 0.01$ by least significant difference test for Group 1.
[e]Significant decrease at $P < 0.01$ by paired *t*-test for Group 2.

TABLE 4

Serum Phospholipid Fatty Acid Composition, Group 1[a]

Fatty acid	Before		Early		Late	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.32	0.08	0.24	0.04	0.19	0.02
16:0	27.55	0.90	30.88	0.50	28.96	0.74
18:0	11.72	0.73	10.10	0.37	9.92	0.46
16:1[b]	0.89	0.19	0.68	0.07	0.59	0.07
18:1[c]	10.20	0.52	9.73	0.48	9.77	0.28
20:3ω9	0.17	0.06	0.09	0.01	0.07	0.01
18:2ω6	18.22	1.61	16.52	1.10	17.75	1.43
18:3ω6	0.09	0.02	0.05	0.00	0.04	0.01
20:2ω6	0.32	0.04	0.20	0.01	0.25	0.02
20:3ω6	3.05	0.76	1.93	0.32	1.95	0.31
20:4ω6	11.22	1.49	13.54	0.56	14.08	0.97
22:4ω6	0.45	0.03	0.42	0.04	0.48	0.04
18:3ω3	0.16	0.02	0.12	0.02	0.13	0.02
20:5ω3	0.58	0.10	0.44	0.03	0.59	0.22
22:5ω3	0.92	0.09	0.80	0.11	1.05	0.10
22:6ω3	3.40	0.54	3.94	0.28	3.81	0.42

[a]Data are wt%, means of n = 5; before, before weight loss; early, first 1–2 mon of very low calorie diet(s) (VLCD); late, 3–5 mon of VLCD.
[b]Sum of 16:1ω9 and 16:1ω7.
[c]Sum of 18:1ω9 and 18:1ω7.

TABLE 5

Serum Free Fatty Acid Composition, Group 1[a]

Fatty acid	Before		Early		Late	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	1.96[d,e]	0.08	1.46[d]	0.08	1.48[e]	0.07
16:0	24.20	0.90	23.75	0.68	22.56	0.79
18:0	8.01	1.17	8.15	0.40	7.90	0.51
16:1[b]	4.19	0.18	3.89	0.36	3.57	0.35
18:1[c]	35.16[e]	1.56	39.82	0.41	41.52[e]	0.59
20:3ω9	0.48	0.27	0.50	0.39	0.27	0.07
18:2ω6	14.64	1.59	13.02	1.33	13.22	1.30
18:3ω6	0.12[f]	0.01	0.09	0.01	0.06[f]	0.01
20:2ω6	0.25	0.01	0.25	0.02	0.31	0.03
20:3ω6	0.28	0.05	0.26	0.02	0.24	0.04
20:4ω6	1.11	0.11	1.04	0.06	0.91	0.09
22:4ω6	0.16	0.02	0.21	0.05	0.17	0.03
18:3ω3	0.82[g]	0.18	0.62	0.15	0.45[g]	0.12
20:5ω3	0.07	0.01	0.05	0.01	0.03	0.01
22:5ω3	0.15	0.02	0.15	0.03	0.11	0.03
22:6ω3	0.42	0.06	0.42	0.07	0.27	0.07

[a]Data are wt%, means of n = 5; before, before weight loss; early, first 1–2 mon of VLCD; late, 3–5 mon of VLCD.
[b]Sum of 16:1ω9 and 16:1ω7.
[c]Sum of 18:1ω9 and 18:1ω7.
[d]$P \leq 0.01$ by analysis of variance (ANOVA).
[e]$P \leq 0.02$ by ANOVA.
[f]$P \leq 0.05$ by ANOVA.
[g]$P \leq 0.03$ by ANOVA.

TABLE 6

Serum Triglyceride Fatty Acid Composition, Group 1[a]

Fatty acid	Before		Early		Late	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	1.65	0.50	0.68	0.06	0.68	0.08
16:0	24.84	1.49	25.51	0.54	24.22	0.52
18:0	3.11	0.41	3.19	0.24	2.83	0.18
16:1[b]	4.95	0.78	3.57	0.21	3.14	0.41
18:1[c]	36.61	0.66	39.80	1.27	40.70	0.99
20:3ω9	0.73	0.56	0.14	0.02	0.21	0.03
18:2ω6	18.52	2.44	18.30	1.57	19.02	1.65
18:3ω6	0.40[d]	0.07	0.27	0.09	0.25[d]	0.10
20:2ω6	0.21[e]	0.03	0.14[e]	0.01	0.18	0.01
20:3ω6	0.30	0.03	0.20	0.02	0.23	0.01
20:4ω6	1.75	0.46	2.17	0.24	2.39	0.33
22:4ω6	0.23	0.04	0.24	0.02	0.27	0.06
18:3ω3	0.76	0.07	0.61	0.13	0.64	0.10
20:5ω3	0.13	0.03	0.12	0.03	0.17	0.02
22:5ω3	0.40	0.08	0.37	0.05	0.49	0.06
22:6ω3	0.49	0.14	0.65	0.10	0.47	0.16

[a]Data are wt%, means of n = 5; before, before weight loss; early, first 1-2 mon of VLCD; late, 3-5 mon of VLCD. Abbreviations as in Table 5.
[b]Sum of 16:1ω9 and 16:1ω7.
[c]Sum of 18:1ω9 and 18:1ω7.
[d]$P \leq 0.03$ by ANOVA.
[e]$P \leq 0.05$ by ANOVA.

was 5- to 20-fold in excess of the minimum amount to avoid EFA deficiency in rats (29) and in humans (30-32). Given this degree of excess provision, there was no decrease in adipose TG 18:2ω6 relative content.

Among the serum fractions, the most notable observation was the decline in free fatty acid 18:3ω3 during the VLCD. Unlike the precursor 18:3ω3, the ω3 product fatty acids EPA and DHA in serum showed no consistent change with the diet. As 18:3ω3 has no known essential functions in its own right other than as a precursor for EPA and DHA, this decline in its level in adipose and in circulatory pools carries no immediate risk. Nonetheless, there could be late sequelae if the 18:3ω3 were not replaced in the post-weight loss phase, as slow equilibration with the hepatic 18:3ω3 substrate pool may impact production of 20:5ω3 or 22:6ω3, allowing their contents in serum or tissues to eventually decline.

Another interesting observation from the serum lipid fraction fatty acid profiles is the low level of 18:2ω6 at all time points as compared to previously published values for lean adults using the same analytical methodology (33). Jones and Schoeller (34) have shown that humans preferentially oxidized polyunsaturated compared to saturated and monounsaturated fatty acids, and that lean and obese subjects differ in their responses to diets varying in polyunsaturated to saturated fatty acid ratio (35). These observations, along with our data suggesting low 18:2ω6 in serum fractions at baseline and during the VLCD, suggest an abnormality in the regulation of 18:2ω6 content in the various serum lipid fractions in obesity. This is consistent with our observations in the obese Zucker rat (36), in nondieting obese humans (37) and in obese humans prior to major weight loss (38). It is clear that the low 18:2ω6 in the three serum fractions did not represent overt EFA deficiency, as PL 20:3ω9 was not elevated.

The mechanism for the selective loss of 18:3ω3 from adipose TG and serum free fatty acids remains unclear. Raclot and Groscolas (39) have shown that 18:3ω3 is preferentially released from stimulated adipocytes, and Clouet *et al.* (40) reported preferential *in vitro* mitochondrial oxidation of α-linolenic acid in the nonesterified form. As the subjects in our study were in marked negative energy balance, dietary 18:3ω3 would be presented to muscle in free fatty acid form, predisposing it to preferential β-oxidation in the mitochondria. This may partially explain the significant reduction in 18:3ω3 in the serum free fatty acid fraction.

The health significance of the reduction in adipose 18:3ω3 with rapid weight loss remains to be determined. One determinant of the physiologic importance of the decline in adipose 18:3ω3 is the extent to which it interacts with other tissue fatty acid pools. Due to the higher rate of adipose fatty acid turnover than oxidation, a reduction in adipose 18:3ω3 will eventually reduce its content in other tissue pools.

Another potential consequence of reduced body ω3 fatty acid stores would be a decrease in the production of EPA and DHA from 18:3ω3. Although this conversion has been reported to be so slow as to be inconsequential (41), other studies indicate that anabolic conversion occurs at a meaningful rate in humans. For example, dietary 18:3ω3 supplementation increases 20:5ω3 content (42-45), and vegan vegetarians do not show depletion of EPA and DHA from serum PL (33). These products of 18:3ω3 modulate cellular response to injury and infection (46,47) and reduce atherosclerosis in an animal model (16) and its manifestations in humans (14,48,49). This participation

of ω3 fatty acids in the pathogenesis of coronary occlusion offers a potential explanation for the findings of Hamm *et al.* (17) and Lissner *et al.* (18), who observed that repeated weight loss in adults predisposed them to early atherosclerosis. If weight loss reduces body ω3 stores independent of the type of fat intake, this may not be subsequently replaced during weight regain if dietary ω3 intake is marginal. Barr *et al.* (21) recently reported that 18:3ω3 constituted 0.3% of calories in a group of young adults given a free choice diet. Thus, a subtle but chronic risk state could be established if recurrent dieting depletes ω3 reserves and intake during maintenance or weight gain does not allow effective repletion. In view of the observation that as few as three fish meals per week (providing a mean of 0.5 g/d of ω3 fatty acids) can reduce atherosclerosis risk (14), this possible explanation for the increased coronary risk with repeated weight loss is not beyond reason.

In conclusion, we have observed a consistent decline in adipose 18:3ω3 content with major weight loss induced by very low calorie dieting. This change occurred independent of the type of VLCD (food or formula) and despite moderate supplementation with 18:3ω3. Both the mechanism through which this fatty acid is selectively removed from adipose tissue and the health implications of this finding deserve further study.

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An Increased Incidence of Apolipoprotein E2/E2 and E4/E4 in Retinitis Pigmentosa

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Previous studies from our laboratory have shown that retinitis pigmentosa (RP), a family of hereditary retinal degenerations, is often accompanied by abnormal levels of cholesterol or polyunsaturated fatty acids. The requirement of the retina for n-3 fatty acids is well known, and a defect in the supply of these lipids (*e.g.*, by apolipoproteins) could affect the course of the disease. The present study confirms and extends a report on apolipoprotein E (apo E) isoforms in German RP patients [Jahn, Oette, Esser, Bergmann, and Leiss, (1987) *Ophthalmic Res. 19*, 285–288] which showed a tenfold increased frequency of the E2/E2 phenotype compared to the average German population. In our study, apo E phenotypes were determined in the probands of 100 Scottish RP families. The findings revealed a 4-fold increase in the incidence of E2/E2 and an 8-fold increase in E4/E4 compared to a Scottish control population. These increases were statistically significant at the $P < 0.05$ and $P < 0.01$ levels, respectively. To investigate the possibility that some of these apparent E2/E2 or E4/E4 phenotypes might actually be new apo E mutations, we examined the behavior of the apo E on sodium dodecyl sulfate–polyacrylamide gels (E2 migrates anomalously) and on isoelectric focusing gels following cysteamine modification of cysteines. These studies showed that two RP patients possibly had new apo E mutations, though amino-terminal sequence analysis revealed no changes in the sequence of the first 19 residues; further sequence analysis is obviously warranted.
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Retinitis pigmentosa (RP) is a family of hereditary retinal degenerations characterized by tunnel vision and night blindness, characteristics which are consistent with the deterioration of rod photoreceptor cells.

Photoreceptor outer segments are highly enriched in polyunsaturated fatty acids and especially in docosahexaenoic acid (22:6n-3, DHA), which comprises as much as 50% of the total fatty acids (1). The continual process of outer segment renewal requires the supply of n-3 fatty acids, in particular DHA, and thus any imbalance might lead to their degeneration. This is supported by evidence from dietary studies on rats and rhesus monkeys where animals deprived of dietary DHA developed visual defects similar to RP (2–5). Therefore it seems possible that the photoreceptor degeneration seen in RP may be due to a defective supply of the appropriate lipids to the retina.

Various lipid defects have been observed in different types of RP. Converse and colleagues (6,7) first reported a trend of hyperlipidemia in RP patients when compared to controls. This was especially noted in men over 35 years of age and hemizygotes of X-linked families. Factors such as diet and lifestyle, which might influence lipid levels, did not differ between the affected and control groups (8). An increase in total serum cholesterol was also noted in a German study of RP subjects, and this was found especially in individuals with autosomal dominant (AD), autosomal recessive (AR) and simplex RP (9). In contrast, hypolipidemia was observed in a number of RP families with AD inheritance (6,10).

In another study, low levels of DHA were found in the plasma of affected X-linked and ADRP patients, compared to their unaffected relatives (6,8,11,12); however, in one AD family, high levels of DHA were reported in the plasma of affected ADRP subjects compared to their unaffected relatives (13). Thus, the pattern of lipid abnormalities appears to vary from one type of RP to another, and this is consistent with the heterogeneity of the disease. However, the question is whether such defects in lipid metabolism play a role in the pathogenesis of some forms of RP.

An investigation of specific categories of lipids may hold the answer. For example, apolipoproteins (apo) play a pivotal role in lipoprotein transportation and metabolism (14–16). Genetic variations of apo E have been shown to be associated with lipid abnormalities; in particular there is an association between the phenotype E2/E2 and type III hyperlipidemia (17). In view of this, Jahn and co-workers (18,19) conducted a survey of apo E phenotypes in the German RP population. A 10-fold increase in the prevalence of E2/E2 was observed in the RP population, in addition to an increase in the heterozygotes E3/E2 and E4/E3, when compared to German controls. This was statistically significant at the $P < 0.001$ level for the E2/E2 homozygote and at $P < 0.05$ for the heterozygote phenotypes, respectively. As apo E2 is a primary gene product, it seems unlikely that its high incidence is secondary to the RP in these patients. The data suggest that the effect on lipid metabolism caused by apo E2 may play a role in the pathogenesis of RP in these patients.

In view of these findings, we investigated the incidence of apo E phenotypes in the Scottish RP population and also the contribution that apo E may have in the etiology of RP.

METHODS

Blood samples were collected in potassium ethylenediaminetetraacetic acid tubes and plasma separated by centrifugation. Apo E phenotyping was performed using the conventional isoelectric focusing of delipidated very low density lipoproteins (VLDL) as described by Wardell *et al.* (20). During the later stages of this study, a recently developed, rapid micromethod by Menzel and Utermann (21) was adopted. This method, based on isoelectric

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Abbreviations: AD, autosomal dominant; AR, autosomal recessive; apo, apolipoprotein; DHA, docosahexaenoic acid (22:6n-3); RP, retinitis pigmentosa; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VLDL, very low density lipoproteins.

focusing of delipidated plasma followed by immunoblotting using polyclonal anti-apo E antibody, has the additional advantage that fresh plasma is not required; it can be performed on stored, frozen plasma.

For the chemical modification studies, the plasma was first treated with cysteamine as described by Weisgraber *et al.* (22). The samples were then subjected to immunoblotting as before.

To determine the apparent molecular weight of apo E, electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) was performed. This method of identifying apo E variants by difference in molecular weight was initially described by Utermann and colleagues (23) who used the SDS-PAGE method of Neville (24). This method was adopted as well as the SDS-PAGE system developed by Laemmli (25).

The amino-terminal sequencing studies were performed as described by Matsudaira (26). This involved directly sequencing proteins that were blotted onto polyvinylidene difluoride membranes which were stained in 0.1% Coomassie blue R250 in 50% methanol. The apo E band was identified, cut out and directly sequenced using the Edman method (27).

RESULTS AND DISCUSSION

The survey of the incidence of apo E phenotypes in the Scottish RP population (Table 1) reveals significant differences when the percentages of the phenotypes are compared to a control Scottish population (taken from a study by Cumming and Robertson, Ref. 28). There is a 4-fold increase in the E2/E2 phenotype but even more striking is the 8-fold increase in the incidence of E4/E4 in the RP population when compared to the controls. The increases in the E2 and E4 homozygotes compared to the other phenotypes were statistically significant at the $P < 0.05$ and $P < 0.001$ levels, respectively, as analyzed by 2×2 Chi-squared tests. This finding is interesting in that the increase in E4/E4 was not previously observed in the German study (18,19). There is very little difference among (control) European populations in the frequencies of the various apo E phenotypes (18-20,28,29).

The significance of this finding to RP is of interest. It is known that both E2 and E4 are "dysfunctional" isoforms in their binding to receptors and catabolism (14,30); the increased prevalence of these isoforms in RP may have some significance in the pathogenesis of the disease.

There are several possible explanations for the above findings. The first is that E2 or E4 might cause some types of RP. This is very unlikely, as the incidence of these isoforms is much greater than the incidence of RP (1 in 2000 or more) (31,32). Secondly, RP might be polygenic or multifactorial, at least in the RP patients who are homozygous for either E2 or E4. That is, the patient would inherit the RP gene, but the disease would only be expressed if they also inherited a "dysfunctional" apo E phenotype such as E2/E2 or E4/E4. This possibility is perhaps consistent with the occurrence of E2/E2 and E4/E4 in simplex cases of RP in both the study by Jahn *et al.* (18,19) and in our study (Table 2).

Another possibility is that there is a new variant of apo E in these RP patients that resembles E2 or E4 but is in fact directly involved either alone or with another gene or factor in the pathogenesis of RP in these patients. This last hypothesis was investigated further.

First, to determine whether the E2 and E4 isoforms from the selected E2/E2 and E4/E4 patients had the predicted number of cysteine residues in their sequence, chemical modification using cysteamine was performed (22). In this method, E2 with two cysteine residues moves to the E4 position (in IEF gels); E3, with one cysteine, also moves to the E4 position, and E4, with no cysteines, does not react with cysteamine and so does not move at all. The isoelectric focusing patterns of the eight E4 homozygotes were the same before and after treatment with cysteamine, indicating that they had no cysteamine residues, as predicted (22). The E2 band in three out of four E2/E2 subjects behaved as expected (Fig. 1), that is, the E2 band moved up two positive charge units to the E4 position, indicating the presence of two cysteine residues. However, in one of the E2 homozygotes (IC) the "E2" band moved up only one charge unit, indicating that this isoform has only one cysteine residue instead of the predicted two residues. The cysteamine result for IC suggests that perhaps this is a variant of the E3 isoform (which has one cysteine residue) and that perhaps there is a substitution elsewhere in the sequence causing the charge difference, thus causing it to focus at the E2 position. This possibility was further investigated by measuring the mobility of the apo E isoforms on SDS-PAGE. The E2 isoform can be distinguished from E3 and E4 by its slower mobility (*i.e.*, higher apparent molecular weight) on SDS-PAGE (23). Figure 2 (method of Laemmli, Ref. 25) shows that two subjects (BH & IC)(lanes c and d)

TABLE 1

Incidence of Apolipoprotein (apo) E Phenotypes in the Scottish Retinitis Pigmentosa (RP) Population Compared to Scottish Controls

	Apo E phenotypes[a]					
	E2/E2	E3/E2	E4/E2	E3/E3	E4/E3	E4/E4
Scottish RP (N = 100)	4[b]	20	1	44	23	8[c]
(%)	(4)	(20)	(1)	(44)	(23)	(8)
Scottish controls (N = 400)[d]	2	51	11	233	99	4
(%)	(1)	(12)	(2)	(59)	(25)	(1)

[a]Apo E phenotypes were determined by isoelectric focusing and immunoblotting (21).
[b]Statistically significant at the level $P < 0.05$.
[c]Statistically significant at the level $P < 0.01$.
[d]Data are from Reference 28.

TABLE 2

Distribution of Apolipoprotein (apo) E Phenotypes in the Scottish Retinitis Pigmentosa (RP) Population by Gender and Type of RP

	Apo E phenotypes[a]						
	E2/E2	E3/E2	E4/E2	E3/E3	E4/E3	E4/E4	Total
Gender							
Male	2	12	1	25	14	3	57
Female	2	8	0	19	9	5	43
							100
Type of RP[b]							
X-L	0	1	0	3	2	0	6
AD	1	7	0	12	4	3	27
AR	0	0	0	1	2	0	3
Mult.	0	1	0	5	3	0	9
Simp.	2	10	1	20	9	5	47
Usher	1	1	0	2	2	0	7
LMBB	0	0	0	1	0	0	1
							100

[a]Apo E phenotypes were determined by isoelectric focusing and immunoblotting (21).
[b]The types of RP are: X-L: X-linked; AD: autosomal dominant; AR: autosomal recessive; Mult.: multiplex; Simp.: simplex; Usher: Usher Syndrome; LMBB: Laurence-Moon-Bardet-Biedl Syndrome.

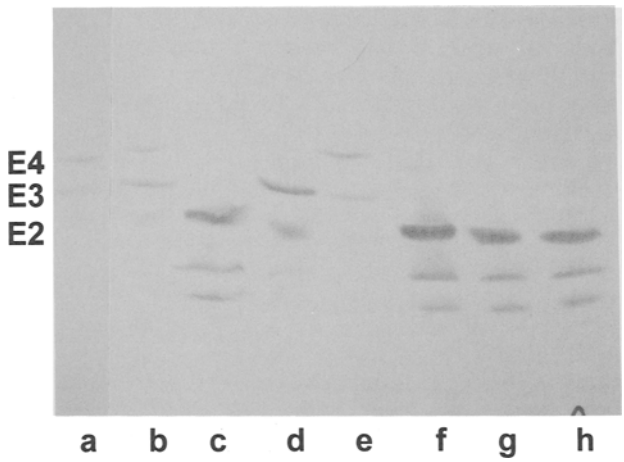

FIG. 1. Immunoblot of delipidated plasma, separated on an isoelectric focusing gel (21), depicting the apolipoprotein E isoforms of four E2/E2 retinitis pigmentosa patients and the effect of cysteamine treatment. The positions of the normal E4, E3 and E2 isoforms are indicated. The vertical lanes contain the following samples: lanes a, f: patient BH, treated (a) and untreated (f), respectively; b, c: patient WA, treated (b) and untreated (c); d, g: patient IC, treated (d) and untreated (g); e, h: patient AP, treated (e) and untreated (h).

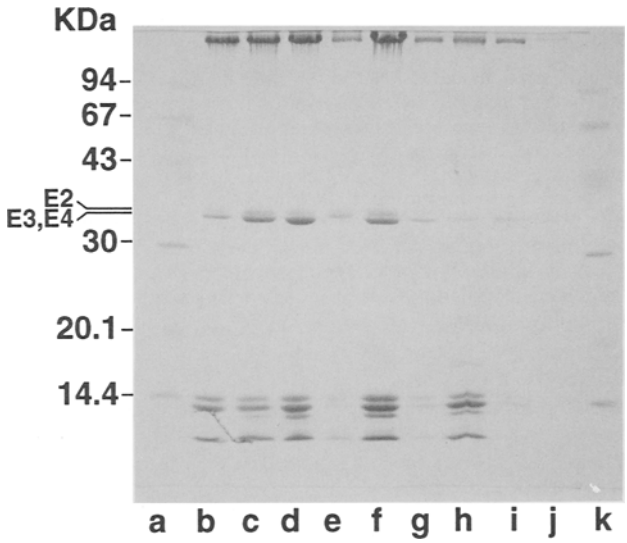

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel (according to Laemmli, Ref. 25) of delipidated very low density lipoprotein (VLDL) from selected retinitis pigmentosa patients, illustrating the mobility of their apo E. Lanes a and k contain low molecular weight protein markers, whose molecular weights in kDa are shown on the y axis. E2, E3 and E4 designate the position of these isoforms. The other lanes are as follows: b: pooled VLDL; c: patient IC (E2/E2); d: patient BH (E2/E2); e: patient AP (E2/E2); f: E3/E2 standard; g: patient LG (E4/E4); h: patient JB (E4/E4); i: patient MS (E4/E4); j: patient HM (E4/E4).

displayed two distinct bands; one band corresponded to the position of E2 and the other to the position of E3 and E4 (which have identical positions). Gels of patients AP and WA (not shown) showed a single band at the E2 position. Similar results were obtained with SDS-PAGE by the method of Neville (24)(data not shown). It is possible that the lower band seen in lanes c and d could be the result of proteolysis; however it is perhaps unusual to get such a strong, distinct band formed by proteolysis, and no such effect was seen in any of the other lanes or in previous studies (23).

The SDS-PAGE results for IC are consistent with the cysteamine modification result that indicated an apo E variant similar to E3. However, BH had two cysteine residues as determined by cysteamine modification (Fig. 1, lane h). It is therefore possible that these two patients have different apo E variants. It has been shown that the E2 variants E2 (Lys 146→Gln) and E2 (Arg 145→Cys) migrate on SDS-PAGE to the positions of E3 and E4 (23), as in our two patients. Therefore, these two patients could have substitutions similar to these variants or could have new variants altogether. The amino-terminal sequences

(up to 19 residues) of these two patients (IC & BH) and two E4 homozygote patients revealed no differences when compared to the predicted amino-terminal sequence (33). Therefore, it seems that if variants exist, the substitutions are further along in the sequence.

The evidence so far points toward two individuals having apo E variants, but the exact positions of the substitutions have yet to be determined.

Apo E has also been found to be synthesized and secreted in other tissues such as kidney, adrenal gland, brain and retina (34,35). Its role in lipid uptake has been demonstrated in photoreceptor cells (36). Growing evidence suggests that apo E plays an important role within the nervous system, for example, in nerve regeneration (16). Although the function of apo E in the retina is still unclear, it is possible that apo E may be involved in the maintenance and repair of the photoreceptor membranes and, if defective, may lead to their degeneration.

In conclusion, if a variant of apo E exists in these patients, it is reasonable to postulate that a defect in lipid metabolism (caused by defective binding of the variant to the receptors) could diminish the supply of lipid to the retina, and this may eventually lead to their retinal dystrophy. Further investigation is warranted to establish the role of apo E in RP.

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Different Resistance of Mammalian Red Blood Cells to Hemolysis by Bile Salts

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To evaluate why hemolysis of red blood cells (RBC) by bile acids varies in different mammalian species, we determined the mean corpuscular volume (MCV), lipid content and the concentrations of the conjugates of deoxycholate and of NaCl inducing 50% hemolysis of RBC from healthy humans, pigs, horses, cows, sheep and jaundiced humans. A volume of 0.05 mL of washed RBC at 1% hematocrit, which has the same lipid content but different phospholipid composition and number of erythrocytes (owing to the variable MCV), was incubated in taurodeoxycholate (TDC) solution (0–5 mM) to determine the TDC concentration inducing 50% hemolysis (TDC_{50}). The TDC_{50} was highest in RBC of sheep and decreased within the series sheep > pig > cow > horse > healthy human > jaundiced human, which have generally increasing MCV. The osmotic resistance followed an inverse order, with jaundiced human > healthy human > horse > cow > pig > sheep. Although we found no correlation between the TDC_{50} and phospholipid composition of the erythrocytes tested, the extent of bile salt-induced hemolysis seemed to depend on both the MCV and the number of erythrocytes in the incubation medium.
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Red blood cells (RBC) from different mammalian species have a remarkably similar lipid content per milliliter of packed cells (1), but their phospholipid composition (1–3), mean corpuscular volume (MCV) (2) and osmotic resistance (4) vary widely. Sphingomyelin (SM) and phosphatidylcholine (PC) are preferentially located in the outer leaflet of the RBC membrane and represent about 45–60% of total phospholipids (PL) (5); their proportions vary in RBC from different animal species (1). Furthermore, the lipid composition of RBC membranes has been considered an important factor influencing the susceptibility to damage from bile salts. Coleman *et al.* (6) have shown that cow and sheep RBC, whose PC content is very low and is replaced by SM, are more resistant to glycocholate-induced hemolysis than human erythrocytes.

In this study we evaluated whether criteria, besides RBC membrane composition, were correlated with bile salt-induced hemolysis of RBC from different species. Variations in the MCV of mammalian erythrocytes may contribute to the different hemolytic effects observed with detergent bile salts.

MATERIALS AND METHODS

Taurodeoxycholate (TDC) and glycodeoxycholate (GDC) (Na^+ salt, grade A) were purchased from Calbiochem (Milan, Italy) and were more than 96% pure as judged by thin-layer chromatography (TLC). Blood from healthy human subjects and from humans with obstructive jaundice, which have large RBC (7), was drawn into heparinized vacutainer tubes. Blood from pigs, cows, sheep and horses was obtained from local slaughterhouses. Cell indices (MCV and mean cell hemoglobin) were evaluated in an S-plus Coulter Counter Analyzer (Coulter, Hialeah, FL). Blood samples were centrifuged and the plasma buffy coat was removed. Then the RBC were washed three times with 15 mM *tris*(hydroxymethyl)aminomethane buffer (pH 7.4) containing 145 mM NaCl and 5 mM glucose. The lipid content of RBC was determined after extracting 1 mL of packed RBC according to Rose and Oklander (8); PL (9) and cholesterol (Biochemia kit no. 676535, Boehringer Mannheim, Mannheim, Germany) were determined on the chloroform extracts. The PL classes contained in RBC membranes were separated by two-dimensional TLC on silica gel G plates (Merck, Darmstadt, Germany), using chloroform/methanol/aqueous ammonia (65:35:5, by vol) as the first solvent and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol) as the second (10). The fractions were located with iodine vapor and scraped off the silica gel plates; PL were measured after digestion with perchloric acid (9). Fractions were identified by comparison with standards (Supelco, Milan, Italy).

The hemolytic effect of TDC and GDC was evaluated as follows: Washed RBC at 1% hematocrit were incubated in TDC and GDC solutions (0–5 mM) in 15 mM of the abovementioned TRIS buffer, pH 7.4, for 45 min, at a final volume of 5 mL. Afterwards, the samples were centrifuged at 5,000 rpm for 4 min (model 153, Microfuge; Beckman Instruments, Fullerton, CA), and the extent of hemolysis was evaluated by comparing absorbance at 546 nm of 100-fold serial dilutions of the supernatant with that of RBC totally lysed in distilled water.

Osmotic fragility of the different RBC was evaluated according to Parpart *et al.* (11). The mean cell fragility was taken as the NaCl concentration (g/dL) at which 50% of the RBC were hemolyzed (12).

RESULTS

Table 1 reports the cell parameters of the RBC tested. The MCV increased along the order sheep, cow, horse, pig and human; the largest RBC came from jaundiced humans (MCV 115 μm^3). Cholesterol and phospholipid content (mg/mL) of the packed RBC was relatively constant in all species, as previously reported by Nelson (1). Even though the lipid content per RBC was greater in humans than in pigs, horses, cows and sheep, the lipid content of RBC from cholestatic humans was even higher (Table 1).

The same volume of packed RBC (0.05 mL) from the various species tested in the hemolysis experiments contained different numbers of cells, even though the total lipid content and the cholesterol to PL molar ratio were roughly the same (Table 2).

Figure 1 shows the hemolysis induced by increasing concentrations of TDC. RBC from sheep, pig and cow were

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Abbreviations: GDC, glycodeoxycholate; MCV, mean corpuscular volume; PL, phospholipid; PS, phosphatidylserine; RBC, red blood cells; SM, sphingomyelin; TDC, taurodeoxycholate; TDC_{50}, TDC concentration inducing 50% hemolysis; TLC, thin-layer chromatography.

TABLE 1

Parameters of Erythrocytes from Different Animal Species[a]

	MCV[b] (μm^3)	S.A.[c] (μm^2)[g]	MCH[d] (pg)	Ch[e] (mg/mL)	PL[f] (mg/mL)	Ch/PL molar ratio	Lipids (g/cell) ($\times 10^{-13}$)[h]
Healthy humans (n = 5)	87 ± 3	142	30 ± 1	1.21 ± 0.3	2.69 ± 0.4	0.91	3.3 ± 0.3
Obstructive jaundice (n = 3)	115 ± 2	160	33 ± 2	1.34 ± 0.2	2.88 ± 0.5	0.94	4.6 ± 0.2
Cow (n = 4)	49 ± 2	90	14 ± 1	1.38 ± 0.3	2.86 ± 0.4	0.97	1.9 ± 0.4
Pig (n = 4)	66 ± 2	95	17 ± 1	1.37 ± 0.3	2.82 ± 0.5	0.98	2.6 ± 0.3
Horse (n = 3)	54 ± 4	83	18 ± 1	1.38 ± 0.2	2.85 ± 0.6	0.98	2.2 ± 0.1
Sheep (n = 3)	25 ± 3	70	12 ± 1	1.39 ± 0.2	2.84 ± 0.4	0.99	1.6 ± 0.1

[a]The values indicate means ± SD.
[b]MCV, mean corpuscular volume.
[c]S.A., surface area.
[d]MCH, mean cell hemoglobin.
[e]Ch, cholesterol; mg/mL of packed red blood cells.
[f]PL, phospholipid; mg/mL of packed red blood cells.
[g]Values were taken from References 12, 13 and 15–17.
[h]Grams of lipids per red blood cell ($\times 10^{-13}$).

much more resistant to TDC than healthy human erythrocytes; the latter cells were less prone to TDC-induced hemolysis than the larger ones that were obtained from jaundiced humans. Horse RBC showed an intermediate behavior. Because the GDC concentrations causing 50% hemolysis overlapped the values reported for TDC, these have not been reported.

At a constant volume of incubated RBC, the TDC concentration causing 50% hemolysis (TDC_{50}) increased in the order sheep > pig > cow > horse > healthy human > jaundiced human (Table 2). The plot of TDC_{50} *vs.* erythrocyte MCV gave a strong negative correlation, which reached statistical significance ($r = -0.83$)($P < 0.02$) (Fig. 2).

At TDC_{50}, the number of bile salt molecules per RBC in the incubation tubes was lowest for horse and highest for pig (Table 2); no correlation was found between this ratio and TDC_{50} ($r = 0.5$; P = not significant).

TABLE 2

Parameters for TDC-Induced Hemolysis[a]

	Number of RBC in the incubation tube ($\times 10^6$)	Lipid content in the incubation tube ($g \times 10^{-7}$)	TDC_{50}[b] (mM)	TDC molecules[c] / RBC number ($\times 10^8$)
Healthy humans (n = 5)	574 ± 42	1894 ± 107	1.38 ± 0.2	71.4 ± 5.4
Obstructive jaundice (n = 3)	435 ± 40	2001 ± 124	1.24 ± 0.2	84.2 ± 6.2
Cow (n = 4)	1064 ± 82	2022 ± 138	2.43 ± 0.3	68.6 ± 4.7
Pig (n = 4)	806 ± 71	2096 ± 99	3.00 ± 0.4	110.8 ± 7.2
Horse (n = 3)	960 ± 68	2112 ± 107	1.89 ± 0.2	58.8 ± 5.0
Sheep (n = 3)	1280 ± 98	2048 ± 111	3.72 ± 0.4	86.0 ± 6.4

[a]The values indicate means ± SD. The final volume of incubation medium was 5 mL (1% Ht).
[b]TDC_{50}: Taurodeoxycholate (TDC) concentration inducing 50% hemolysis.
[c]Number of TDC molecules per erythrocyte in the incubation medium at TDC_{50}. RBC, red blood cells.

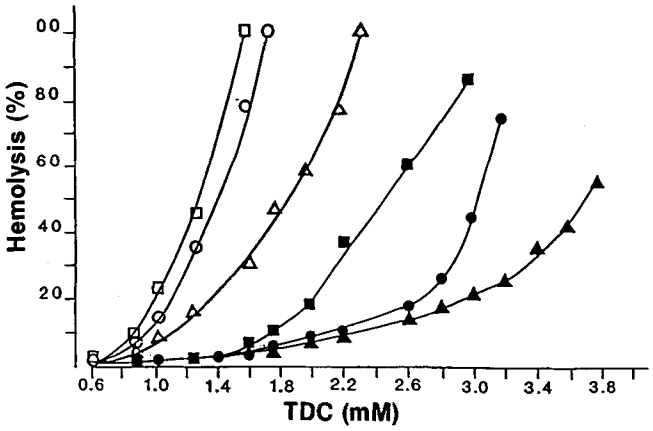

FIG. 1. Hemolysis of red blood cells from healthy human (○), cholestatic patient (□), cow (■), sheep (▲), pig (●) and horse (△), induced by increasing concentrations of taurodeoxycholate (TDC). The data shown are the means of three experiments. The final volume of the incubation medium was 5 mL (1% Ht).

TABLE 3

NaCl Concentrations Inducing 50% Hemolysis[a]

	NaCl (g %)	MCV (μm^3)
Healthy humans (n = 5)	0.44 ± 0.05	87 ± 3
Obstructive jaundice (n = 3)	0.41 ± 0.06	115 ± 2
Cow (n = 4)	0.57 ± 0.08	49 ± 2
Pig (n = 4)	0.62 ± 0.10	66 ± 2
Horse (n = 3)	0.52 ± 0.08	54 ± 4
Sheep (n = 3)	0.63 ± 0.07	25 ± 3

[a]The values indicate means ± SD. MCV, mean corpuscular volume.

Hemolytic behavior also seems to be correlated with the physical characteristics of the erythrocytes studied. The osmotic resistance of RBC from different mammalian species decreased with MCV (Table 3). The correlations between the NaCl concentration inducing 50% hemolysis and both MCV ($r = -0.86$) ($P < 0.001$) and TDC_{50} ($r = 0.95$, $P < 0.003$) were statistically significant.

The RBC of the species studied had variable amounts of choline-containing PL (Table 4), as reported by Nelson (1,3). In particular, SM accounted for more than 50% of total PL in cow and sheep RBC, whereas PC content was near zero in these species. In contrast, SM proportions are similar in human, pig and horse RBC, even though the latter two have smaller MCV and a higher resistance to TDC than human RBC. The correlation between SM content of RBC membranes (expressed as percent of total PL) and the TDC_{50} was not statistically significant (Fig. 3).

DISCUSSION

Previous studies have indicated that RBC containing SM are more resistant to bile salt-induced hemolysis (6). Several hematological parameters, such as MCV, surface area and surface-to-volume ratio differentiate erythrocytes from different mammalian species (12–17); surface area is negatively correlated with the volume of the RBC (18). Hypotonic stress reduces the surface-to-volume ratio, and hemolysis occurs after the cells have become spherical and have reached a critical volume. Osmotic resistance is strongly correlated to the surface-to-volume ratio of the RBC (7,18), and it increases with MCV (4,12) (Table 3). An increment of both the surface area and the surface-to-volume ratio, as occurs in cholesterol-enriched RBC of cholestatic humans, enhances osmotic resistance (7) (Table 3); these erythrocytes are resistant to hemolysis from hypotonic stress but are susceptible to the action of deoxycholic acid conjugates. By contrast, erythrocytes with

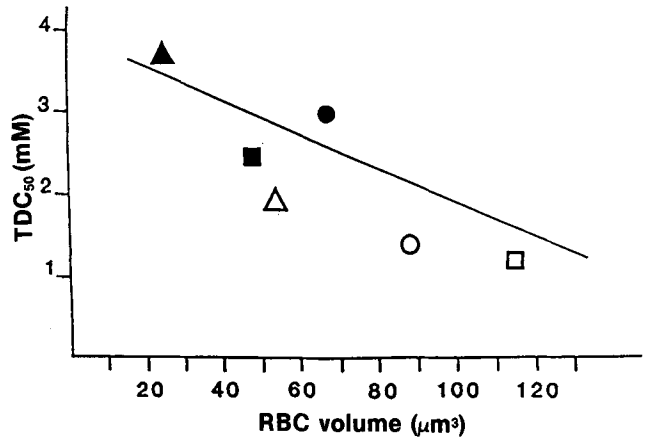

FIG. 2. Correlation between taurodeoxycholate (TDC) concentrations able to induce 50% hemolysis (TDC_{50}) and mean corpuscular volume of the red blood cells (RBC) tested (μm^3) ($r = -0.83$, $P < 0.02$). Symbols indicate RBC from healthy human (○), cholestatic human (□), cow (■), sheep (▲), pig (●) and horse (△).

TABLE 4

Phospholipid Composition of Red Blood Cells from Various Animal Species[a]

	(% of total phospholipids)						
	LPC	PI	PS	SM	PC	PE	Other
Healthy humans	1.6	1.8	12.9	25.7	39.5	26.2	3.1
Obstructive jaundice	1.3	1.8	12.6	23.4	32.4	26.1	2.2
Cow	—[b]	2.1	15.9	50.8	1.5	26.8	2.7
Pig	0.9	2.7	16.1	20.1	30.3	28.2	1.6
Horse	1.2	1.2	16.2	20.1	34.3	25.0	1.9
Sheep	—[b]	1.8	14.2	56.2	1.6	24.4	1.6

[a]The data were taken from three healthy humans, two humans with obstructive jaundice and three animals each for cow, pig, horse and sheep. LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.
[b]—, Not detected.

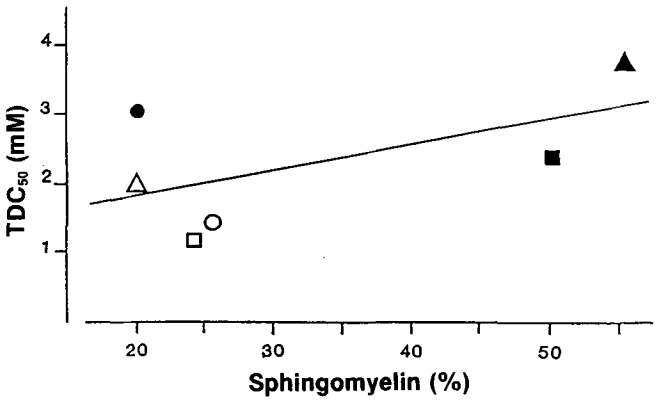

FIG. 3. Correlation between taurodeoxycholate (TDC) concentration inducing 50% hemolysis (TDC_{50}) and the percentage of sphingomyelin in red blood cell (RBC) membranes (r = 0.60; P = N.S.). Symbols indicate RBC from healthy human (○), cholestatic patient (□), cow (■), sheep (▲), pig (●) and horse (△).

small surface-to-volume ratio, such as those from sheep and cow, are osmotically fragile but resistant to bile salts (19). Erythrocytes with small MCV would lyse at lower bile salt concentrations if cell swelling alone were responsible for breaking down the cell membrane. Instead, the smaller cells are more resistant to the detergent effect of tauroconjugates of deoxycholic acid (Table 2) (Fig. 2), even if the number of TDC molecules per erythrocyte required to obtain 50% hemolysis does not vary accordingly (Table 2).

Few data are available on the mechanisms underlying hemolysis induced by bile salts; at appropriate concentrations bile salts solubilize RBC membrane constituents (20), but like other anionic drugs, sublytic concentrations of bile salts actually protect against hypotonic stress (21). Hypotonic hemolysis and hemolysis induced by bile salts thus occur *via* different mechanisms.

Bile salt hemolysis is traditionally evaluated by incubating packed RBC with serial concentrations of bile salts (6). In our hemolysis experiments, 0.05 mL of packed RBC contained a variable number of cells but the same amount of lipids (Table 2); therefore the volume of packed erythrocytes used in the hemolysis experiments contained more cells when these had a small MCV, as for sheep, pig and cow.

As the concentration of TDC required to determine 50% hemolysis was inversely correlated with the volume of the erythrocytes tested (r = −0.83; see Fig. 2), it seems that some physical characteristics of the single erythrocyte play a role in determining the extent of hemolysis in the presence of TDC.

The variable resistance to damage by bile salts has been attributed to the peculiar PL composition of RBC from some animal species (2,6). In fact, SM largely replaces phosphatidylcholine in cow and sheep RBC (1) (Table 4). SM-rich membranes are typically less fluid and are lysed more slowly by bile salts than are more fluid membranes (19). Moreover, SM reduces the permeability to water (22) and phosphates (23). The influx of glycerol, phosphate and urea decreases when the ratio of poly- to monounsaturated fatty acids in the membrane decreases (4,5), as is the case in pig and sheep RBC (23,24).

The correlation between TDC-induced hemolysis and SM percentage in RBC membranes was not statistically significant (Fig. 3). Schubert and Schmidt (25) demonstrated *in vitro* that SM does not stabilize membranes against bile salt damage, as suggested by Lowe and Coleman (19) for erythrocytes. The latter authors incubated RBC at a concentration of 2% (by vol), so that the number of cells changed widely for the different animal species tested. Support for the importance of additional factors besides lipid composition comes from the hemolytic behavior of RBC from pig and horse; the erythrocyte PL composition in these species compares well with that in humans, but pig and horse RBC are smaller and have a TDC-resistance similar to those rich in SM.

In conclusion, several factors, including membrane characteristics and PL composition (6), influence the resistance of RBC to bile salts. In this study we have shown that both the MCV and the number of erythrocytes present strongly influence the extent of bile salt-induced hemolysis.

ACKNOWLEDGMENT

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Fatty Acid Content and Composition of *Oenothera hookeri* Seeds Containing Mutant Plastids

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The effects of plastid mutations on seed oil content and fatty acid composition are of considerable interest. Seeds of a number of plastome mutants produced by the *pm* plastome mutator line of *Oenothera hookeri* were therefore harvested to investigate these effects. The mutants were altered solely in their plastome: each seed lot had the same nuclear background. To facilitate the study, a rapid single-step method was developed to simultaneously assay both oil content and fatty acid composition of small quantities of *Oenothera* seed. The lipid analyses showed that the mutated plastome often changed the oil content of the seeds, and that such changes always reduced oil content. Strong negative correlations were observed between oil content and palmitate or γ-linolenate, and a strong positive correlation was observed between oil content and linoleate. This is the first instance to our knowledge in which plastid mutations have been unequivocally demonstrated to affect seed oil content and composition. Such effects would be indirect, since the mutated plastid genes would not be the structural genes for enzymes on the pathway of oil biosynthesis. The plastid mutations demonstrate another layer of potential complexity in understanding oilseed genetics.
***Lipids* 28, 1005–1009 (1993).**

Analysis of the inheritance of oil content in seeds of diverse species, such as rapeseed (1), soybean (2,3), sunflower (4) and lupin (5), has shown that seed oil content is determined largely by the genotype of the maternal parent and that the effect of the genotype of the seed embryo is small. By contrast, seed oil composition is largely under the control of the genotype of the developing embryo with smaller and variable maternal influences. For example, in soybean, maternal influences have been reported to influence oleate and linoleate levels (3), but not linolenate (3,6) or stearate levels (7). Maternal control of seed oil content in rapeseed does not persist into the F2 generation and appears not to be under cytoplasmic control (1). It was therefore reasoned that maternal control of oil content was mediated by a metabolic dependence of the developing embryo on the seed-bearing plant (8). However, it has been suggested that other factors, such as genetic cytoplasmic elements or even endosperm tissue, might play a role in seed oil biosynthesis (5). The effects of cytoplasmic inheritance on fatty acid composition and content are documented for corn (9). Sixteen male sterile cytoplasms (presumably mutations in the mitochondrial genome) were examined in two nuclear backgrounds. The oleate and linoleate levels were similar in the different cytoplasms, while oil content varied considerably.

Oenothera has recently received considerable attention as a producer of γ-linolenic acid (10–12). *Oenothera biennis* is grown commercially for seed production. Another facet of the genus *Oenothera* is the availability of plastome mutants generated by plants homozygous for the recessive gene *pm*, plastome mutator (13). This gene functions similar to other nuclear gene-induced plastid mutator systems (14) and includes the induction of plastid mutations with many different phenotypes (13,15). The precise function of *pm* is not known, but it is believed to be involved in chloroplast DNA replication or repair (16).

Chloroplast mutants are maintained as mosaics on plants with normal green plastids. *Oenothera* has biparental transmission of plastids, and mutant plastids can be transmitted sexually if flowers develop on stems which have mutant plastids in the subepidermal histogenic tissue layer LII, since the pollen and egg cells are derived from this tissue (17). When a flower which derives from LII with mutant plastids is self-pollinated, all the seeds are composed of tissues with mutant plastids only. By identifying variegation on sibling plants within a population derived from the self-pollination of a single inbred plant, the nuclear genetic influences can be kept constant, and one can study the differences generated by the plastid mutations.

Most, if not all, of the genes coding for the enzymes of fatty acid and lipid synthesis are nuclear encoded (18). Also, it is known that the site of *de novo* fatty acid biosynthesis in plants is the plastid (19). Thus, it is of interest to see how plastid mutations would affect seed oil content and composition in *Oenothera*, since the mutations would not directly affect enzymes on the pathway. To facilitate this study, a rapid, single-step method was developed to simultaneously assay the oil content and fatty acid composition of small quantities of *Oenothera* seeds.

MATERIALS AND METHODS

Plant material. Plants of an inbred line (84007) of *O. hookeri* strain Johansen with plastome I were used as the control for this study. Plants of an inbred line (84009) of *O. hookeri* strain Johansen with plastome I and homozygous for the gene *pm* were used as the source of plastome mutations. During the course of the growing season, over 100 of the 411 sibling plants within 84009 developed variegated sectors. Once a mutational sector was evident, the sector remained stable in color and enlarged in a manner consistent with the patterns of growth and development characteristic of *Oenothera*. A sample of sectors was selected for analysis, which typified the range of possible mutant phenotypic colors. The photosynthetic lesions of these mutant chloroplasts were not determined. All experimental plants were grown together in a greenhouse to minimize environmental factors that might alter oilseed content and composition. Seed from self-pollinated flowers originating from 17 different sectors that included mutant subepidermal histogenic layer II were harvested. Additionally, the seeds of two green plants were harvested individually for use as controls. Paired comparisons were made with two plants where seeds were produced both on an all-green branch and on a mutant sector. Also compared were two mutants on a single plant. For both types of paired comparisons, analyses will reflect plastid differences, because the nuclear contribution is identical.

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Abbreviation: GC, gas chromatography.

For the analysis of tissues with plastome mutations, the seeds were from individual plants or from individual sectors on single plants. Because the nuclear genetic background of these sibling inbred plants were similar, the differences between mutants is thought to reflect the influence of the chloroplastic mutants themselves.

Lipid analysis. Seeds were weighed into 50–100 mg lots, dried to constant weight at 80°C and reweighed. The seeds were transferred to glass reaction tubes with Teflon-lined screw caps, and the derivatization reagent was added. This reagent was composed of 2.0 mL of 5% sulfuric acid in methanol and 1.0 mL of either benzene or toluene containing a triheptadecanoin internal standard at a concentration of 0.5 mg/mL. The seeds plus reagent were heated at 90–100°C for 4 h, cooled, and 3 mL of water added. The tubes were shaken vigorously and then centrifuged to break the emulsion and give phase separation. An aliquot of the upper phase was injected directly onto the gas chromatography (GC) column.

GC analysis was performed using a 30 m × 0.25 mm fused silica column coated with Supelcowax 10 (Supelco, Bellefonte, PA) stationary phase, run isothermally at 245°C, with injector and flame-ionization detector temperatures set at 250°C, using a "splitless" injection mode. Peak areas were measured with an electronic integrator. Because the variation in fatty acid chain length is very limited, fatty acid quantitation used peak areas without applying any corrections.

Multiple Soxhlet extractions of ground seeds with hexane were used to determine seed oil content. Linear regression analyses were performed on the seed oil content and fatty acid composition data set to determine r^2 values.

RESULTS AND DISCUSSION

Determination of fatty acid content and composition. Because of the limited amount of seed tissue available from the *pm* mutants, in some cases between 0.1–0.2 g, routine methods of oil content analysis (by nuclear magnetic resonance and gravimetrically by oil extraction with organic solvents) were considered inappropriate for accurate and quantitative measurements. However, it seemed reasonable that the small seeds of *O. hookeri* (*ca.* 0.45 mg each) could be derivatized directly in the presence of an internal standard, allowing the derivatization reagent to also perform the organic extraction. A similar approach was used to rapidly screen leaf lipids for fatty acid composition (20). Although the use of such direct derivatization methods on plant tissue to determine fatty acid composition is well known, their use to determine fatty acid content has only recently been demonstrated (21).

Soxhlet extraction of control seeds gave 0.247 mg oil per mg seed. Transmethylation of this oil in the presence of an internal standard gave 0.214 mg fatty acids per mg seed. For crushed seeds, the derivatization/extraction was rapid (< 30 min), but for whole seeds it was also complete within two hours. The final values for the extraction/derivatization protocol with crushed or whole seeds were slightly higher than for the extracted oil. Six independent determinations of fatty acid content gave 0.217 ± 0.003 mg fatty acids per mg seed (Table 1, Experiment 1). This indicated that a high degree of reproducibility is possible for this type of analysis. Fatty acid composition of the extracted oil differed only marginally from that obtained by the derivatization/extraction protocol. The comparison was repeated in a second experiment (Table 1, Experiment 2). The slightly higher fatty acid contents compared to Experiment 1 may have resulted from a new batch of triheptadecanoin internal standard being made up, the quality of the internal standard being a potential source of error between experiments.

The results reported from Experiments 1 and 2 (Table 1) are in general agreement with those reported in the literature for *O. hookeri* (10). The small differences could easily be due to either genotypic differences within the species, or environmental differences during plant growth and seed set (22). The method can thus be used to accurately, rapidly and simultaneously screen for both fatty acid composition and oil content in small batches of dried seeds.

Analysis of the fatty acid content and composition of Oenothera *seeds.* Table 2 shows the seed weights and seed lipid analyses for 17 different mutants and five controls ("normal green") of *O. hookeri.* The sectors producing these seeds were considered independent plastome mutational events, because the sectors were not evident in the cotyledonary tissues and, in most cases, not in the first few leaves of the maternal plants. The colors of the sectors ranged from "white" to "yellow" to "light green" and "yellow green". The frequency of the mutational events,

TABLE 1

Comparison of Methods for Analysis of Seed Fatty Acid Composition and Content in *Oenothera hookeri*

Extraction method	Number of replications	Fatty acid content (mg/mg seed)	Fatty acid composition (%)				
			16:0	18:0	18:1	18:2	18:3[a]
Experiment 1							
Soxhlet	1	0.214	5.50	2.03	5.41	76.77	10.28
Derivatization	6	0.217 ± 0.003	5.74 ± 0.14	2.12 ± 0.06	5.44 ± 0.08	76.78 ± 0.60	9.91 ± 0.65
Experiment 2							
Soxhlet	3	0.225 ± 0.001	5.48 ± 0.02	2.00 ± 0.01	5.34 ± 0.02	77.11 ± 0.04	10.07 ± 0.04
Derivatization	6	0.228 ± 0.004	5.66 ± 0.09	2.08 ± 0.04	5.37 ± 0.10	76.81 ± 0.25	10.08 ± 0.44
Taken from Reference 10							
Soxhlet	1	0.241	6.3	2.4	9.3	74.0	7.0

[a]18:3 is solely γ-linolenic acid.

TABLE 2

Weight, Fatty Acid Content and Fatty Acid Composition of *Oenothera hookeri* (strain Johansen) Seeds with Normal or Mutant Plastids

Plant identification number	Phenotype of LII	Average weight per seed (mg)	Number of replications[a]	Fatty acid content (%)[b]	Fatty acid composition (%)					18:3 Content (%)[b,c]
					16:0	18:0	18:1	18:2	18:3[c]	
Controls										
84007	Normal green	0.440	9	22.7	5.8 ± 0.1	2.3 ± 0.0	5.6 ± 0.1	75.6 ± 0.2	9.5 ± 0.1	2.16
Bulked seeds										
84007-4	Normal green	0.400	3	20.4	6.2	2.1	5.6	75.2	9.9	2.02
84007-5	Normal green	0.445	3	24.1	5.6	2.1	5.5	75.9	9.6	2.31
Paired comparisons										
84009-213 green × 84007-6										
	Normal green	0.505	3	26.1	5.8	2.3	6.0	75.5	9.4	2.45
84009-213	Yellow	0.445	3	20.6	6.8	2.3	5.1	71.8	13.0	2.68
84009-20	Normal green	0.425	3	24.3	6.1	2.7	5.9	73.4	10.6	2.58
84009-20	Light yellow	0.410	3	24.3	6.0	2.6	5.4	74.1	11.1	2.70
84009-389	Yellow green	0.396	3	18.6	7.7	2.2	4.8	68.6	15.4	2.86
84009-389	White	0.360	3	16.9	7.7	2.4	7.9	66.8	13.4	2.26
Additional mutants										
84009-353	Light green	0.455	2	25.6	5.9	2.4	5.1	74.3	11.2	2.87
84009-348	Light yellow	0.440	3	25.3	5.9	2.1	4.3	74.7	11.8	2.99
84009-42	Light yellow	0.450	3	24.4	6.0	2.7	5.5	74.1	10.8	2.64
84009-110	Light yellow	0.415	3	23.9	6.1	2.5	5.5	73.8	11.1	2.65
84009-302	Yellow	0.400	3	23.8	5.8	2.2	4.7	74.4	11.7	2.78
84009-87	Mottled	0.475	2	23.3	6.5	2.5	5.2	73.7	10.8	2.52
84009-394	Mottled	0.500	3	22.5	6.7	2.7	5.6	72.8	10.9	2.45
84009-129	White	0.430	3	22.4	6.4	2.7	5.8	72.8	11.3	2.53
84009-154	Yellow green branch	0.370	3	18.9	7.3	1.9	4.2	72.0	13.6	2.57
84009-18	White	0.410	3	18.4	7.0	2.7	8.1	67.9	13.0	2.39
84009-179	White	0.405	3	14.6	7.8	2.8	10.2	65.2	12.4	1.81
84009-38	White	0.410	3	14.0	8.0	2.4	7.4	66.2	14.5	2.03
84009-139	Yellow branch	0.155	2	6.2	10.3	1.7	4.3	65.3	15.9	0.99

[a]Number of seed batches analyzed for fatty acid composition and content. For each seed batch, duplicate gas chromatography runs were made.
[b]Total fatty acid or γ-linolenic acid contents are expressed as mg fatty acids per mg dry seed weight × 100.
[c]18:3 is solely γ-linolenic acid.

the spectrum of phenotypic differences and the cytoplasmic transmission of the variegation were consistent with published data (13). Although the mutational frequency of plants homozygous for *pm* is approximately 1,000 times higher than spontaneous rates (13), the rate is still sufficiently low that only rarely do two mutations occur on a single plant within a single growing season. Thus the sectors were expected to give results that would be consistent even when sexually transmitted to subsequent generations. In Table 2, oil content is reported on a dry weight basis. The seed weights ranged from 0.36 to 0.50 mg per seed, indicating good seed fill in all cases except for 84009-139. In 84009-139, the seeds were uniformly smaller and had much lighter colored seed coats. The sector for this mutant involved a whole branch and also showed reduced seed weight. The other mutants were maintained as periclinal chimeras in which LII had the mutant plastids, and LIII was normal green. The tissues with mutant plastids derived their energy from the normal green portions of the plant. This juxtaposition of mutant and green tissues may influence the response of the mutants. Some examples of the influence of adjacent tissue have been described in *Oenothera* (23) involving nuclear-plastome incompatibilities. However, since the seeds with mutant plastids differ from the seeds with wild-type plastids, this influence may cause some modulation in response but the influence was insufficient to compensate for the defects in the mutant plastids.

The oil content of the bulked seeds was 22.7%, and the range for seeds from individual normal green plants was 20.4 to 26.1%. The range of oil content values for the seeds with mutant plastids was 6.2 to 25.6% with 10 of the 17 seed lots from mutant plastid sectors falling within the wild-type range. No plastid mutations were observed which increased oil content.

The results of three paired analyses are presented in Table 2. In two comparisons, seeds from both the normal green portion of the plant and seeds from a mutant sector on the same plant were analyzed. In both single plant comparisons, the seeds in the green portion were a little heavier. In the 84009-213 comparison, the fatty acid content of the seeds from the green tissue exceeded the content of the seeds from mutant tissue, but in the other example (84009-20) the fatty acid content was equivalent. Mutant seeds from 84009-213 had a significantly higher percentage of γ-linolenic acid than seeds from the "normal green" sector control. The third paired analysis involved two different plastid mutations on one plant, 84009-389. Both mutations had average seed weights below 0.4 mg/seed, with fatty acid contents below 20%, and both had γ-linolenic acid above 13%. Overall, the paired analyses show the uniqueness of each plastome mutation when the environmental factors and the nuclear genetic influences are kept constant.

Seeds from 13 additional mutant sectors were analyzed without a green paired control. The results of these analyses are listed in Table 2 in order of decreasing oil content. There was a strong inverse correlation between palmitate and oil content ($r^2 = 0.926$). Seeds from green control tissues had a palmitate range of 5.6–6.2%, while seeds from mutant tissues ranged from 5.9 to 10.3%. The inverse relationship between palmitate and oil content is interesting in the light of the current emphasis on breeding oilseeds for reduced saturated fatty acid levels. Other significant trends were an increase in γ-linolenate ($r^2 = 0.629$) and a decrease in linoleate ($r^2 = 0.781$) as the fatty acid content decreased, and an inverse relationship between linoleate and γ-linolenate ($r^2 = 0.700$). For linoleate, seeds of control tissues ranged from 73.4 to 75.9%, and the seeds with mutant plastids ranged from 65.3 to 74.7%. The ranges for γ-linolenate were 9.5 to 10.6% for the controls and 10.8 to 15.9% for the seeds from mutants. The changes in fatty acid composition are a reflection of the fatty acid composition of the triacylglycerol fraction (data not shown), which is the dominant lipid in the seed oils of all the mutants.

The changes in the level of γ-linolenic acid are of particular interest because, in *Oenothera*, γ-linolenic acid is a seed-specific fatty acid. It is reported to be absent in leaves (12). The biosynthesis of γ-linolenic acid is believed to occur by the cytoplasmic desaturation of *sn*-2-linoleoyl phosphatidylcholine and has been most extensively studied on *Borago officinalis* (24). The Δ6 desaturase is presumably coded by a nuclear gene, as plastid-encoded gene products are not exported to the cytoplasm. We can speculate that the plastome-mutant modulation in the relative amount of γ-linolenic acid might be controlled by the flux through the lipid biosynthetic pathway, since there appears to be an inverse relationship between the amounts of linoleic and γ-linolenic acid. A reduced flux would result in a longer residence time for linoleoyl moieties in phosphatidylcholine molecules, increasing their availability for Δ6 desaturation. In some of the mutants, not only does the percentage of γ-linolenic acid in the oil increase, but the actual content of γ-linolenic acid on a percentage of seed weight basis also increases. Clearly, further experiments to test lines such as 84009-389, -348 or -353 for the inheritance of increased γ-linolenate and for seed yield would be worthwhile. Conversely, breeders selecting for higher γ-linolenate in *Oenothera* seed oils should be aware of the inverse linkage between γ-linolenate and oil content, and the potential influence of cytoplasmic (plastid) genetics; they may prefer to make selections to break this linkage and to look for nuclear-encoded traits.

The implication is that in some general way these mutant plastids have a genetic defect which prevents the plastids from developing into fully functional, photosynthetically active chloroplasts. This represents the visual basis upon which the mutants were identified and selected. The results in Table 2 would indicate that these lesions appear also to affect the accumulation and processing of fatty acids in seeds. The plastid mutations involved in this study have not been analyzed for their fine structure using electron microscopy. However, four other *pm*-induced mutants have been analyzed (15). In these four mutants, the internal membranes were reduced in quantity in phenotypes with less color. In particular, mottled mutants had developed disorganized membranes, yellow had a few too many abnormal thylakoids, and white had little to no internal membranes. A recent analysis of leaf tissues from a specific *O. hookeri* plastome mutant, *pm7*, showed that approximately equal amounts of plastid membrane components (fatty acids, acyl lipids and carotenoids) were present in both mutant and wild-type (25). Instead, there appeared to be a defect in chloroplast protein processing, which resulted in a lack of photosynthetic electron transport chain activity, thylakoid membrane

structures and chlorophyll biosynthesis. Fatty acid biosynthesis was not affected and was apparently not tightly linked to the development of a functional photosynthetic apparatus.

For fatty acid synthesis in seeds, the *pm*-induced mutations involve biosynthetic activity, which precedes the need for fully developed chloroplasts since only leucoplasts (proplastids) exist in developing seeds during the time when the seeds oils are synthesized. However, the present study has demonstrated that it is possible for mutations in the plastid genome to significantly alter seed oil content and modulate seed oil composition. This is not a surprising result, but we believe that this is the first time such a phenomenon has been directly demonstrated. It is not clear exactly what the influence of pigment-deficient maternal tissue is on the change in oil accumulation in the developing embryo and whether or not the plant adjusts its seed set to compensate for the reduced photosynthate. Indeed, the simplest explanation is that the changes in oil composition and content are mediated through reduced photosynthate availability in the supporting maternal tissues. However, the paired comparisons do strongly suggest that the mutants themselves are a major determinant in both accumulation and composition.

The effects of these plastome mutations on seed oil content and composition are almost certainly indirect; that is, the mutated genes do not code for enzymes directly involved in lipid biosynthesis. However, these results highlight the effect of an additional genetic component, the plastid, which can bring additional complexity to our understanding of seed oil genetics. Whether the observation that plastid mutations only decrease oil content, and the relationships between oil content and palmitate or linoleate are universally applicable to all oilseeds, remain to be determined, as do the mechanisms underlying these effects.

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Isolation of Brominated Long-Chain Fatty Acids from the Phospholipids of the Tropical Marine Sponge *Amphimedon terpenensis*

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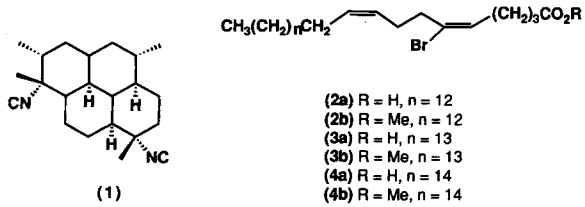
Preliminary investigation of the phospholipid fatty acid composition of the tropical marine sponge *Amphimedon terpenensis* by gas chromatography/mass spectrometry revealed the presence of some novel brominated fatty acids. Two new brominated fatty acids, (5*E*, 9*Z*)-6-bromo-5,9-tetracosadienoic acid (2a) and (5*E*, 9*Z*)-6-bromo-5,9-pentacosadienoic acid (3a) were subsequently isolated from a chloroform/methanol (3:1, vol/vol) extract of the sponge and characterized as their methyl esters 2b and 3b. The known brominated fatty acid (5*E*, 9*Z*)-6-bromo-5,9-hexacosadienoic acid (4a) was also isolated. The new fatty acid methyl esters were confirmed as brominated Δ5,9 acid derivatives by chemical ionization mass spectrometry. The position of the bromine substituent was determined to be C-6 by nuclear magnetic resonance techniques while the stereochemistry of the two double bonds was deduced by nuclear Overhauser enhancement difference spectroscopy. The biosynthetic implications of the co-occurrence of the three brominated acids are discussed.

Lipids 28, 1011-1014 (1993).

Marine sponges (Phylum porifera) are characterized by wide variation in sterol composition and by the presence of nuclear-modified or side chain alkylated sterols (1,2), some of which are cell membrane components (3,4). The unusual composition of marine sterols is frequently complemented by the presence of unusual phospholipid fatty acids (1,5), and is presumed to result in a unique membrane structure which may be an important factor in the survival of this "dead end" phylum. Characteristics of the fatty acids isolated from sponges include long carbon chains (C_{24}-C_{34} very long chain fatty acids, VLFA) with novel branching and unsaturation patterns or substituents (1,6,7).

The tropical marine sponge *Amphimedon terpenensis* contains low levels of Δ5,7 sterols with conventional side chains (8), together with large quantities of a tetracyclic diterpene, diisocyanoadociane **1** (Scheme 1), recently shown by us to be associated with the sponge cell membranes (9). This raised the intriguing possibility that the phospholipid components of this sponge might be modified in order to accommodate the terpene within a stable lipid bilayer, which prompted us to analyze the fatty acids present in *A. terpenensis;* we now report the structures of two new brominated fatty acids characterized as their methyl esters using nuclear magnetic resonance (NMR) and mass spectrometric (MS) analysis.



SCHEME 1

EXPERIMENTAL PROCEDURES

Sponge samples. Specimens of *A. terpenensis* (Australian Museum No. 4978) were collected near Townsville using scuba, specifically from John Brewer Reef at −15 m in November 1987 and at −10 m from Davies Reef in December 1989.

Extraction and isolation of phospholipids. A. terpenensis (10 g) collected in November 1989 was extracted with $CHCl_3$/methanol (1:1, vol/vol) to yield total lipids (350 mg). The neutral lipids, glycolipids and phospholipids were separated according to the procedure of Privett *et al.* (10). A portion of the phospholipid extract (50 mg) was transmethylated with 1.4 M HCl in methanol and the resulting fatty acid methyl esters (FAME) purified by passage through a short plug of florisil (200 mesh; Aldrich, Milwaukee, WI) using toluene as eluant. After solvent removal, the residue was dissolved in hexane and analyzed by capillary gas chromatography (GC) using a Hewlett-Packard (Palo Alto, CA) 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, and an automatic injector system (sampler model 7672A), injector (model 3392A) sampler/event control module (model 19405A), together with a computerized fatty acid library (software S/N 2614R100027, version 1.2) previously used for the fatty acid analysis of *Pseudaxinyssa* sp. (11), *Tethya* sp. (12) and a hymeniacidonid sponge (13).

Isolation and identification of fatty acids. A specimen of *A. terpenensis* (380 g wet weight) collected in November 1989 was washed in sea water and carefully cleaned of all non-sponge debris and chopped into small pieces. After freeze-drying, the sponge was extracted with $CHCl_3$/methanol (1:1, vol/vol) yielding a total of 15.6 g of lipids (4.1%). A portion (3.12 g) was transmethylated with 14% BF_3/MeOH under N_2, and the FAME were purified by silica column chromatography using hexane/diethyl ether

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Abbreviations: CIMS, chemical ionization mass spectrometry; COSY, correlation spectroscopy; ECL, equivalent chain length; EIMS, electron impact mass spectrometry; FAME, fatty acid methyl ester; FTIR, Fourier transform infrared; GC, gas chromatography; HMQC, heteronuclear multiple quantum coherence; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; VLFA, very long chain fatty acid.

(19:1, vol/vol) as eluant to give 50 mg of FAME extract. A 1-mg portion of FAME was dissolved in 0.25 mL hexane and passed through a short silica column (thin-layer chromatography grade, 5 cm height silica) eluting with hexane (10 mL). After solvent concentration, the resulting hexane extract (1 mL) was analyzed by GC. For the isolation and characterization of individual FAME, the remaining extract (49 mg) was first purified by reverse-phase high-performance liquid chromatography (HPLC) (Waters Millipore, Bedford, MA; M6000 pump, U6K injector, R401 differential refractometer) using two Altex Ultrasphere ODS columns (10 mm i.d. × 25 cm) in series and absolute methanol as the mobile phase at 3 mL/min. Fractions identified by GC as containing brominated FAME were further purified by reverse-phase HPLC using two Altex Ultrasphere ODS columns (10 mm i.d. × 25 cm) and acetonitrile/methanol/ethyl acetate (11:4:4, by vol) as mobile phase at 2 mL/min to give methyl (5*E*, 9*Z*)-6-bromo-5,9-tetracosadienoate (**2b**; HPLC retention time in MeOH relative to methyl palmitate, 1.97; 0.50 mg, 1.0%), methyl (5*E*,9*Z*)-6-bromo-5,9-pentacosadienoate (**3b**; HPLC retention time in MeOH relative to methyl palmitate, 2.29; 1.75 mg, 3.5%), and methyl (5*E*,9*Z*)-6-bromo-5,9-hexacosadienoate (**4b**; HPLC retention time relative to methyl palmitate 2.70; 0.65 mg, 1.3%), respectively. Further processing of the crude extract provided 2.1 mg of **2b**, 10.6 mg of **3b** and 2.7 mg of **4b**. *N*-Acyl pyrrolidide derivatives were prepared by direct treatment of methyl esters with pyrrolidine/acetic anhydride (10:1, vol/vol) in capped vials at 100°C for 1 h, followed by ethereal extraction from the acidified solution and purification by short column chromatography using hexane/diethyl ether (8:2, vol/vol) as eluant. Samples for ^{1}H NMR were run as solutions in $CDCl_3$ on a Varian XL-400 (Palo Alto, CA), JEOL GX-400 (Tokyo, Japan) or a Bruker AMX-500 spectrometer (Karlsruhe, Germany), referenced to $CHCl_3$ at δ 7.25. Samples for ^{13}C NMR were run as solutions in $CDCl_3$ on a Bruker AMX-500 spectrometer with Waltz decoupling referenced to $CDCl_3$ at 77.0 ppm. A 2D COSY (correlation spectroscopy)-45 spectrum was run on a Varian XL-400 spectrometer using a spectral width of 2946 Hz, an acquisition time of 0.17 s and a pulse delay of 1.0 s. Sixteen scans were accumulated for each of 256 increments into each of 1024 data points. The spectrum was zero-filled before Fourier transformation. Nuclear Overhauser enhancement (NOE) difference spectra were obtained on a JEOL GX-400 spectrometer using a 90° pulse and a 20 s pulse delay. A heteronuclear multiple quantum coherence (HMQC) experiment was run on a Bruker AMX-500 spectrometer using 8 scans for each of 512 increments accumulated into 2048 data points, with a 0.6 s delay for the Bird Sequence, and 3 s recycle delay (14). The experiment was optimized for a ^{13}C-^{1}H one-bond coupling constant of 135 Hz. The phase cycling program TPPI was used. Prior to Fourier transformation, the initial data matrix was zero-filled in each dimension and multiplied by a sine-squared bell function in both the t_1 and t_2 dimensions. The spectrum was not symmetrized, but the T_1 noise was eliminated using the program *Aurelia*. Samples for electron impact mass spectrometry (EIMS) were dissolved in hexane and run on a Hewlett-Packard HP 5995 GC/MS instrument in the direct insertion mode while samples for chemical ionization mass spectrometry (CIMS) were run on a Ribermag R-10-10 quadrupole mass spectrometer with ammonia as reagent gas. GC/MS samples were analyzed using a Ribermag GC/MS/DS system which combines a Ribermag R-10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractionvap chromatograph (Carlo Erba, Milano, Italy) containing a fused silica column (28 m × 0.32 mm) with SE-54 (J&W Scientific Inc., Folsom, CA). Samples for Fourier transform infrared (FTIR) spectrometry were run on a Bomen DA3.26 spectrometer (Bomen, Quebec, Canada) as solutions in $CHCl_3$.

A FAME extract of a hymeniacidonid sponge was prepared by $CHCl_3$/MeOH treatment of wet tissue (21.2 g) followed by methylation as described above.

RESULTS

The major fatty acids of *A. terpenensis* were identified in standard fashion from GC equivalent chain length (ECL) values and EIMS of methyl esters and *N*-acyl pyrrolidides. As shown in Table 1, the most abundant phospholipid fatty acids were generally 16:0 and 18:0 together with 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid); however, the three brominated acids, initially detected by GC/MS, were also present in significant quantity. Complete phospholipid fatty acid analyses of *A. terpenensis* will be presented in a separate paper (15).

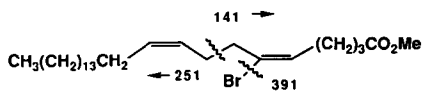
Samples of each new brominated FAME were purified by reverse-phase HPLC and their structures elucidated by NMR and MS. Under CIMS conditions, the major brominated FAME, **3b**, had strong peaks due to ($M + NH_4^+$) adduct ions with the 51:49 ratio characteristic of a single bromine substituent. The molecular ion (*m/z* 472, 470) corresponded to a molecular formula of $C_{26}H_{47}O_2Br$, confirming a C_{25} acid, while major peaks at 391, 251 and 141 were assigned as shown in Scheme 2. Under EIMS conditions, **3b** showed a major peak at *m/z* 74 (McLafferty rearrangement) together with a series of peaks at *m/z* 391, 359, 341 and 317, closely corresponding to those for a C_{26} monobrominated FAME **4b** recently reported from

TABLE 1

Major Phospholipid Fatty Acids of *Amphimedon terpenensis* by Analytical GC[a]

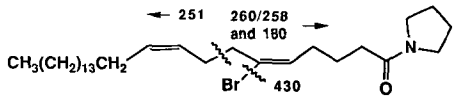
FAME	ECL	%
16:0	16.00	13.5
18:0	18.00	2.6
Phytanic acid	17.74	10.0
24:2 + Br (**2b**)	26.24	0.7
25:2 + Br (**3b**)	27.28	2.5
26:2 + Br (**4b**)	28.31	1.1
18:1	17.76	2.1

[a]Normalized to phytanic acid, average of three values. Other fatty acids present in *A. terpenensis* are identified in Reference 15. Abbreviations: GC, gas chromatography; FAME, fatty acid methyl esters; ECL, equivalent chain length; Br, brominated.



SCHEME 2

a hymeniacidonid sponge (13), except for the 14 amu mass difference. EIMS of the pyrrolidide derivative of **3b** gave weak molecular ions at 511 and 509 in the expected ratio, together with ions at 430, 260, 258, 180 (assigned as in Scheme 3) and 113 (McLafferty rearrangement). This mass spectrum corresponded to that reported for the pyrrolidide of **4b** apart from the 14 amu difference.



SCHEME 3

The structure of **3b** was unambiguously elucidated by 400 MHz NMR in $CDCl_3$. Downfield signals in the spectrum were a triplet (1H) at δ 5.85 together with a complex 2H multiplet at δ 5.31–5.41. The methylene envelope at δ 1.25–1.40 integrated for 26 protons while the presence of a triplet integrating for 3H at δ 0.87 ruled out chain branching. The connectivity from $(H\text{-}2)_2$ at δ 2.32 (*t*) to H-5 at δ 5.85 was deduced from a COSY-45 spectrum as was that from $(H\text{-}7)_2$ at δ 2.45 (*t*) to $(H\text{-}11)_2$ at δ 2.06 (*m*). In particular, $(H\text{-}7)_2$ and H-5 showed only one cross peak each in the COSY-45; therefore, the bromine substituent was located at C-6, consistent with the downfield shift of the adjacent H-5 relative to H-9 and H-10. Full ^1H and ^{13}C assignments are given in Table 2.

The absence of prominent bands between 960–980 cm^{-1} in the FTIR spectrum of **3b** suggested a *Z* configuration for the Δ9 double bond (13,16). This stereochemistry, together with the *E* stereochemistry for the Δ5 double bond, were confirmed by the observation of NOE effects between H-4 and H-7 and between H-8 and H-11. The 5*E*, 9*Z* stereochemistry has been found previously in brominated fatty acids (13,16).

The second new brominated FAME was suggested by its GC and HPLC characteristics to be the C_{24} acid **2b**, and this identification was confirmed by NMR, MS and HPLC. Under EI conditions, **2b** showed the expected series of peaks at 377, 345, 327, 303, 236, 141 and 74. The *N*-acyl pyrrolidide showed weak molecular ions at 497 and 495 together with ions at 416, 260, 250, 180 and 113. The proton NMR spectrum was identical to that of **3b** except that the methylene envelope integrated for 24 protons. The lack of prominent IR bands between 960–980 cm^{-1} suggested a *Z* configuration for the Δ9 double bond.

The remaining brominated FAME **4b** was identified as the C_{26} acid by comparison of its GC, MS and NMR data with the compound reported recently by Lam *et al.* (13). The stereochemistry of the two double bonds of **4b** was confirmed as 5*E*, 9*Z* by co-injection of a purified sample of **4b** from *A. terpenensis* with a crude extract prepared from a hymeniacidonid sponge.

TABLE 2

Proton and ^{13}C Nuclear Magnetic Resonance (NMR) Data for Brominated Fatty Acid Methyl Esters (2b)–(4b)[a]

	Compound			
	2b	**3b**	**3b**	**4b**
	NMR data			
No.	$^1H^b$	$^1H^b$	$^{13}C^{c,d}$	$^1H^b$
1	—	—	173.6	—
2	2.32 (7.4)[e]	2.32 (7.4)[e]	33.2	2.32
3	1.71 (7.4)[e]	1.73 (7.4)[e]	24.4	1.71 (7.4)[e]
4	2.06	2.06	28.9	2.06
5	5.84 (7.7)[e]	5.85 (7.6)[e]	131.7	5.84 (7.8)[e]
6	—	—	126.3	—
7	2.45 (7.6)[e]	2.45 (7.5)[e]	35.6	2.45 (7.6)[e]
8	2.30	2.29	25.8	2.30
9	5.32	5.31	127.3	5.32
10	5.41	5.41	131.5	5.41
11	2.06	2.06	27.3	2.06
12–23	1.20–1.40	1.25–1.40	29.7	1.20–1.40
24	0.87			
25	—	0.87	14.1	
26	—	—	—	0.88
OMe	3.67	3.67	51.5	3.67

[a]Solution in $CDCl_3$.
[b]^1H at 400 MHz.
[c]^{13}C at 125 MHz.
[d]Protonated signals assigned by heteronuclear multiple quantum coherence spectroscopy (14).
[e]*J* values in parentheses.

DISCUSSION

Litchfield and Morales (17) reported high levels of long-chain fatty acids in sponges of the class Demospongiae, and furthermore suggested that these "demospongic" fatty acids might be membrane components. Bergquist and colleagues (18,19) reported on the fatty acid composition of 85 species of Porifera and noted that seasonal and geographic variations occur, restricting the chemotaxonomic relevance of fatty acid data. Brominated fatty acids have to date been found in *Xestospongia* spp. (C_9, C_{16} and C_{18} acids), in *Petrosia* spp (C_{18}, C_{27} and C_{28} acids) and in a sponge tentatively identified as belonging to the family Hymeniacidonidae (C_{26} acid) (13,16,20–27). *Xestospongia* and *Petrosia* represent very closely related genera with similar skeletal morphology, internal structure, growth form and ecology. It is now agreed that both genera belong within the family Nepheliospongidae (order Nepheliospongida). The precise taxonomic status of the sponge used in this study is unclear; it may prove to be a new genus, certainly within the order Haplosclerida, but the presence of isonitrile metabolites is reminiscent of some Axinellida. Until further electron microscopy can be done on cell structure, the sponge is referred to the genus *Amphimedon* (family Niphatidae, order Haplosclerida) (28). It is possible that the presence of brominated fatty acids may represent a useful taxonomic characteristic with which to position sponges in the Nepheliospongida and Haplosclerida. The species sample is too small at the present to make any strong suggestion on that matter.

Fatty acids with a Δ5,9 substitution pattern are characteristic of marine sponges (2,5,7,8,13,16). The VLFA isolated in this study have exclusively Δ5,9 unsaturation. There is no evidence for the presence of trienoic acids such as 24:3 or 26:3 (2,13) or the corresponding monoenoic acids 24:1, 25:1 and 26:1 (2,11–13) in *A. terpenensis*. Although Carballeira and Shalabi (27) recently reported the isolation of C_{27} and C_{28} brominated fatty acids from a Petrosiid sponge, the isolation of 24:2 brominated, 25:2 (brominated)

and 26:2 (brominated) represents an interesting example of such homology in a marine sponge.

The biosynthesis of brominated fatty acids has been evaluated by Lam *et al.* (13) and was found to conform to the currently accepted mode of fatty acid biosynthesis in marine sponges (1). The bromine substituent is introduced following chain elongation and desaturation of a 16:0 precursor. In this manner, the formation of 24:2 brominated and 26:2 brominated acids may be explained, but our isolation of a 25:2 brominated acid suggests that sponges may additionally have the capacity to chain extend, desaturate and functionalize an odd carbon chain length fatty acid also. Odd carbon chain fatty acids, such as 15:0 and 17:0, generally have a branched structure rather than straight chain structure and are usually found in bacteria. *A. terpenensis* contains both bacterial and cyanobacterial symbionts (10), thus an additional consideration is which of the three cell types, sponge, bacteria or cyanobacteria contains the brominated fatty acids. This question is being addressed in another report from our group (15).

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Oxidations of the α-Tocopherol Model Compound 2,2,5,7,8-Pentamethyl-6-chromanol. Formation of 2,2,7,8-Tetramethylchroman-5,6-dione

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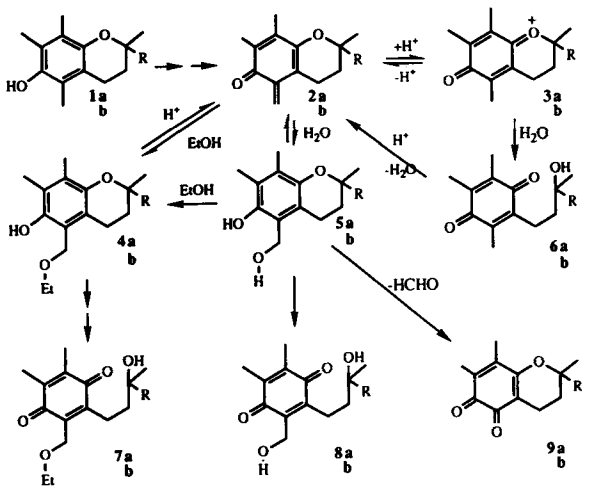
Overoxidation of α-tocopherol (1a) by silver nitrate produces tocored (9a) as a major product. The aim of the present work was to elucidate the pathway of formation of tocored using the α-tocopherol model compound, 2,2,5,7,8-pentamethyl-6-chromanol (1b). Oxidation of 1b by silver nitrate in ethanol produces 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b) and 2,2,7,8-tetramethylchroman-5,6-dione (9b, the model compound of tocored) as major products. Formation of 6b is rapid and is accompanied by an equally rapid fall in pH. Formation of 9b only occurs after 6b has reached maximum concentration and has begun to decline. It appears that acid promotes the dehydration and recyclization of 6b into a quinone methide (2b), which is then rehydrated into 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5b), the phenolic isomer of the quinone 6b. Oxidative deformylation of 5b leads to 9b. It is also demonstrated that 6b, heated in ethanol in the presence of acid and in the absence of any oxidizing agent, is converted into 9b, 1b, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b) and 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (7b). It seems that dehydration and recyclization of 6b into 5b occurs as above and that 6b then oxidizes 5b into 9b, while being reduced into the hydroquinone of 6b (6bH_2). Compound 6bH_2 then cyclizes in acid to 1b. A possible alternative pathway from 6b to 9b that does not involve 5b is also discussed. These results suggest that 6b and, by implication, α-tocopheryl quinone (6a), is not a stable compound and, in the presence of acid, is readily oxidized to 9b.

Lipids 28, 1015–1020 (1993).

There has been much discussion in the literature as to whether α- or γ-tocopherol is the better antioxidant. Much of the early work suggested that γ-tocopherol (**10a**) was a better antioxidant than α-tocopherol (**1a**) (1–4), whereas more recent work has suggested the reverse (5–8). It has been proposed that antioxidant activity may be best assessed in an initiated autoxidation system and that, in such a system, **1a** is better than **10a** (9). Using an uninitiated autoxidation system, we have found that 2,2,7,8-tetramethyl-6-chromanol (**10b**), the model compound of **10a**, was a far superior antioxidant to 2,2,5,7,8-pentamethyl-6-chromanol (**1b**), the model compound of **1a** (10). Thus our results agree with those in the early literature.

We have suggested that the superior performance of **10b** over **1b** in the uninitiated system may be due to oxidation products of **10b** rather than to **10b** itself (10). Thus 2,2,7,8-tetramethylchroman-5,6-dione (**9b**), which is a major oxidation product of **10b** (Kohar, I., and Southwell-Keely, P.T., unpublished results) has antioxidant activity and may extend the activity of **10b**, whereas 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**6b**), which is the major oxidation product of **1b**, has no antioxidant activity (10).

Compound **9b**, in addition to being a major oxidation product of **10b**, can also be formed by overoxidation of **1b** (11). Treatment of **1b** with silver nitrate at 65°C in absolute ethanol for 2 h gave **9b** (40%) and 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**4b**, 30%) as the major products (12). Formation of both these compounds is believed to occur through an intermediate quinone methide (**2b**) (Scheme 1). Addition of ethanol to **2b** would form **4b**, whereas addition of water to **2b**, followed successively by oxidation, further addition of water, deformylation and further oxidation would form **9b** (12). Although this reaction was performed in absolute ethanol, there was sufficient water (0.5%) in the solvent to account for formation of compound **9b** by this pathway. Nonetheless, there was an overwhelming excess of ethanol in the reaction, and this led to the thought that compound **4b** might also be an intermediate in the formation of **9b**. The earliest report of compound **9b**, which did not include characterization of its structure, indicated that it could be formed from **1b** and also from **6b** by silver nitrate oxidation (11). Although **6b** was not a significant product



SCHEME 1

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Abbreviations: EI, electron impact; HPLC, high-performance liquid chromatography; IR, infrared; MS, mass spectrum; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; UV, ultraviolet.

of our silver nitrate oxidation of **1b**, it was decided to follow the rate of oxidation of compounds **4b**, **5b** and **6b** in turn with silver nitrate to determine the most probable pathway to **9b**.

MATERIALS AND METHODS

Infrared (IR) spectra were determined on a Perkin Elmer (Norwalk, CT) 580B spectrometer, ultraviolet (UV) spectra on a Perkin Elmer 124 double beam spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (Karlsruhe, Germany) AM 500 spectrometer and were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard. Electron impact (EI) mass spectra were determined at 70 eV on an AEI (Manchester, England) MS 12 spectrometer. pH Measurements were made on a Crison micropH 2001 pH meter (Crison Instruments, Barcelona, Spain).

Compound **1b** (13), together with its spirodimer (14) and spirotrimer (14), 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (**4b**) (15), 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (**5b**) (16), 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (**6b**) (11), 5-formyl-2,2,7,8-tetramethyl-6-chromanol (17), 1H-2,3-dihydro-3,3,5,6,9,10,11a(*R*)-heptamethyl-7a(*S*)-(3-hydroxy-3-methylbutyl)pyrano[2,3-a]xanthene-8(7aH),11(11aH)dione (18), 2-(3-hydroxy-3-methylbutyl)-3-hydroxymethyl-5,6-dimethyl-1,4-benzoquinone (**8b**) (16) and 2,2,7,8-tetramethylchroman-5,6-dione (**9b**) (12), were prepared as reference compounds by known methods.

Hexane (Ajax Chemicals, Auburn, Australia) and isopropanol (BDH Chemicals, Poole, England) for high-performance liquid chromatography (HPLC) were passed through an Activon (Sydney, Australia) 0.45-μm filter, degassed (Waters Associates pump, Waters Associates, Milford, MA) and sonicated (Branasonic 12, Branson, Danbury, CT) before use. HPLC employed a Waters Associates M-45 solvent delivery system, U6K injector, with a 2-mL loop, a model 440 fixed wavelength (280 nm) detector, a National Pen Recorder (Matsushita, Yokohama, Japan) VP-6513A and an SIC Chromatocorder 12 (Alphatech, Tokyo, Japan) integrator. All compounds other than **5b** were separated isocratically using hexane/isopropanol (95:5, vol/vol) on a Waters μPorasil column (10 μm; 300 × 3.9 mm) at a flow rate of 1.1 mL/min. Separated compounds had the following retention times (min): **4b**, 3.0; **1b**, 3.4; **6b**, 5.1; **7b**, 6.3; **9b**, 7.2; and **8b** 14.0. For reasons that are not clear, but have to do with the lability of the compound, **5b** could not be detected with this system or in other normal phase systems (19), but was detected using methanol/water (92.5:7.5, vol/vol) as eluent on a Lichrosorb C_{18} column (10 μm; 250 × 4.6 mm) (Alltech Associates, Deerfield, IL). Compounds **5b**, **6b** and **9b** have the same retention time (3.7 min) in this reverse-phase system. Therefore, in order to determine the concentration of **5b**, duplicate samples were analyzed by normal- and reverse-phase HPLC, as above. The concentrations of **6b** and **9b**, determined by normal-phase HPLC, were converted into the corresponding areas for the reverse-phase system, and these were subtracted from the reverse-phase peak, which contained all three compounds.

Oxidation procedure. The following oxidation procedure was applied to compounds **1b**, **4b**, **5b** and **6b** and is illustrated for compound **1b**.

Oxidation of **1b** *by silver nitrate in absolute ethanol.* Compound **1b** (50 mg) was dissolved in absolute ethanol (45 mL) and heated to 60°C. An aliquot (2 mL) was taken as a control, then silver nitrate (500 mg) and absolute ethanol (5 mL) were added. The solution was stirred at 60°C for 3 h, during which it became orange-red and silver precipitated. Aliquots (2 mL) were taken for analysis at the following intervals (2, 5, 10, 15, 30, 60, 120 and 180 min). Each aliquot was diluted with water (5 mL) and extracted with diethyl ether (3 × 5 mL). The combined ether extracts were washed with water (2 × 5 mL), dried (Na_2SO_4) and the solvent removed *in vacuo.* The dry residue was dissolved in a little light petroleum and dried again under nitrogen. All fractions were analyzed quantitatively by HPLC.

pH of the reaction between **1b** *and silver nitrate.* Compound **1b** (25 mg) was dissolved in absolute ethanol (25 mL) and an aliquot (3 mL) taken immediately, diluted with distilled water (7 mL), and the pH determined. Silver nitrate (250 mg) was added to the remaining solution (22 mL), and the solution was heated at 60°C. Aliquots (3 mL) of the reaction mixture were taken at various intervals (1, 2, 5, 10, 20, 30 and 45 min) and added to cold distilled water (7 mL) to slow the reaction. The solutions were allowed to come to room temperature for pH determination. The pH values shown in Figure 1 are those of the diluted solutions. No attempt was made to correct the pH for dilution as the nature of the acidic material was unknown.

pH of the reaction between **4b** *and silver nitrate.* The protocol for this reaction was exactly the same as for the reaction between **1b** and silver nitrate.

Oxidation of **6b** *by silver nitrate in absolute ethanol.* Compound **6b** (100 mg) was dissolved in absolute ethanol

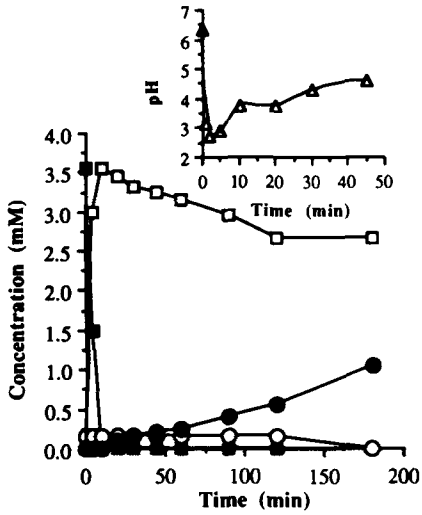


FIG. 1. Formation of products in the reaction between silver nitrate and 2,2,5,7,8-pentamethyl-6-chromanol (1b) in absolute ethanol at 60°C; ■, 1b; ○, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b); □, 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b); ●, 2,2,7,8-tetramethylchroman-5,6-dione (9b). Insert shows the change in pH during the course of reaction.

(20 mL), heated to boiling and an aliquot (1 mL) was taken as a control. Silver nitrate (1 g) was added, and the solution was stirred under reflux for 3 h. After 50 min the color turned orange, and after 60 min red crystals appeared on the wall of the flask. Aliquots (1 mL) of the reaction mixture were taken for HPLC analysis at the following intervals (5, 10, 15, 30, 60, 90, 120 and 180 min). The aliquots were diluted with water, extracted with diethyl ether and analyzed by HPLC as described previously.

Reaction of **6b** *with 0.03M sulfuric acid in absolute ethanol.* Fifty mg of **6b** was added to a 0.03-M solution of sulfuric acid in absolute ethanol (10 mL), and the solution heated at 60°C under nitrogen. Aliquots (1 mL) of the reaction mixture were taken for HPLC analysis at the following intervals (10, 20, 30, 45, 60 and 90 min). One mL of brine was added to each aliquot, and the solution extracted with diethyl ether (3 × 1 mL). The combined ether extracts were washed with brine (5 × 1 mL) and distilled water (1 mL), dried (Na_2SO_4) and the solvent removed *in vacuo*.

Reaction of **6b** *with* **5b** *in absolute ethanol containing 0.001 M sulfuric acid.* To a 0.001 M solution of sulfuric acid in absolute ethanol (5 mL) was added **5b** (25 mg) and **6b** (25 mg), and the solution heated at 60°C. Aliquots (0.5 mL) of the reaction mixture were taken for HPLC analysis at the following intervals (3, 5, 15, 30, 45 and 60 min). One mL of brine was added to each aliquot, and the solution extracted with diethyl ether (3 × 1 mL). The combined ether extracts were washed with brine (5 × 1 mL) and distilled water (1 mL), dried (Na_2SO_4) and the solvent removed *in vacuo*.

*Isolation of 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (***7b***).* Compound **4b** (50 mg) and silver nitrate (500 mg) were added to absolute ethanol (50 mL), and the solution heated at 60°C for 3 h during which time the solution changed to blood red and silver precipitated. Water (25 mL) was added, and the solution was extracted with diethyl ether (3 × 25 mL). The combined ether extracts were washed with water (3 × 25 mL), dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was chromatographed on thin layers of silica gel GF_{254} [solvent, petroleum hydrocarbon (b.p. 60-80°C)/ethyl acetate, 1:1, vol/vol]. The fractions were located under UV light and eluted with distilled chloroform. The solvent was removed under a stream of nitrogen. Major bands with the following R_f values were isolated: 0.26 (yellow, 13.4 mg), 0.39 (yellow, **6b**, 12.6 mg), 0.51 (**9b**, 15.7 mg), 0.65 (colorless, changing to yellow-orange on thin-layer chromatography; unidentified, 5.5 mg), 0.82 (yellow; unidentified, 1.2 mg). The yellow band with R_f 0.26 was identified as 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (**7b**) and had the following spectral data: UV (λ_{max}, hexane) 260, 265 (sh) nm; IR(KBr) (cm^{-1}): 3437 (OH), 2975, 2932, 2875, 1648 (C=O), 1467, 1449, 1379, 1304, 1279, 1237, 1219, 1153, 1124, 1099 (C-O-C), 1030, 937, 910, 877, 761, 727. MS (EI) *m/z* (rel. intensity): 280 (M^+) (2), 262 (7), 234 (33), 221 (50), 176 (100). ^1H NMR ($CDCl_3$): δ 1.20 (*t*, 3H, J = 7.0 Hz, -O-CH_2-CH_3), 1.25 (*s*, 6H, 2 × CH_3), 1.62 (*m*, 2H, =C-CH_2-CH_2), 2.01 (*s*, 6H, 2 × =C-CH_3), 2.67 (*m*, 2H, =C-CH_2-), 3.55 (*q*, 2H, J = 7.0 Hz, -O-CH_2-CH_3), 4.40 (*s*, 2H, =C-CH_2-O-); ^{13}C NMR ($CDCl_3$): δ 187.77, 186.72, 148.48, 140.88, 140.69, 138.32, 70.84, 66.81, 62.60, 43.17, 29.18 (2 peaks), 21.98, 15.25, 12.45, 12.36.

RESULTS

Figure 1 shows that compound **1b** was oxidized completely to **6b** in 10 min, after which the concentration of **6b** fell gradually, but it was still the major product (70 mole %) after 180 min. Compound **9b** did not appear until 10 min and then rose gradually to 28% at 180 min. Compound **4b** rose rapidly to 5% within 5 min and then fell very slowly to zero at 180 min. The product distribution in this present silver nitrate oxidation of **1b** differed a great deal from that observed previously (12). The major product by far in the present reaction was compound **6b**, which was only observed in very small amounts in the previous reaction (12). By contrast compound **4b**, which had been one of the major products in the previous reaction (12), was only a very minor product in the present reaction. The reason for this product reversal may be the amount of water in the reaction. Thus the molar ratio of water/**1b** in the present reaction (16:1) was much greater than in the previous study (3:1). It has been shown that a relatively low concentration of water in the presence of a large excess of ethanol favors the formation of **6b** rather than **4b** (20).

The fact that compound **9b** did not appear until **1b** had disappeared completely suggests that **9b** was a secondary product. Also, compound **9b** only began to form after **6b** had reached a maximum and was beginning to decline. This suggested that **9b** was being formed from **6b**. In order to test this theory, **6b** was heated at 60°C and, subsequently, at 78°C in ethanol in the presence of silver nitrate, but was found to be stable and unchanged after several hours. The original report of the formation of **9b** indicated that it could be formed from **6b**, as well as from **1b** (11). The lack of reactivity of **6b** in our reaction was in conflict with the original report until it was realized that the original authors had used a much more concentrated solution of **6b** (13 mg/mL) than we had used (1 mg/mL). On changing to a more concentrated solution (5 mg/mL) and heating at 78°C, we observed the results shown in Figure 2. The reaction showed a lag period of almost one hour before any change occurred, after which **6b** was converted fairly rapidly into **9b**. The reason for this lag period is unknown. As **6b** at a concentration of 1 mg/mL was

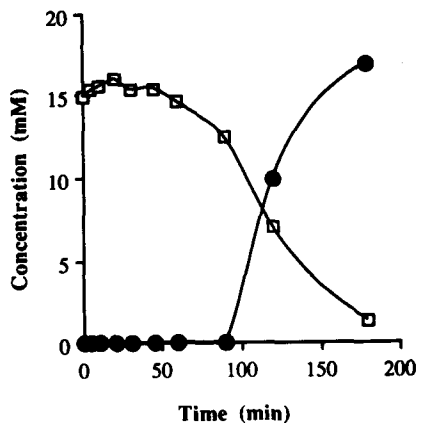


FIG. 2. Formation of 2,2,7,8-tetramethylchroman-5,6-dione (9b) from the reaction of silver nitrate with 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b) in absolute ethanol at 78°C; □, 6b; ●, 9b.

stable when heated directly with silver nitrate at 78°C, but decomposed steadily at 60°C after it was formed in the **1b** oxidation (Fig. 1), it appeared that another product of the **1b** oxidation might be promoting the decomposition of **6b**. A study of pH change during **1b** oxidation revealed an initial, rapid drop to pH 2.7, followed by a much slower rise (insert, Fig. 1). A pH of 2.7 corresponds closely to that which would have been obtained if **1b** had dissociated completely. However, as **1b** is a very weak acid, these results suggest that silver nitrate reacts with **1b** to liberate a proton as follows:

$$Ag^+ + ArOH \rightarrow Ag + ArOH^{+\bullet} \rightarrow ArO\bullet + H^+ \qquad [1]$$

This pH drop was definitely due to reaction between silver nitrate and **1b**, because a solution of silver nitrate in ethanol had a pH of 6.5.

In order to determine the effect of acid on the course of reaction, **6b** was heated at 60°C under nitrogen in the presence of 0.03 M sulfuric acid and in the absence of silver nitrate. Figure 3 shows that the concentration of **6b** fell by 40% within 2 min and then by a further 30% in the next 88 min. Compound **6b** was converted into **4b**, **1b**, **7b** and **9b**. When this reaction was repeated using 0.03 M hydrochloric instead of sulfuric acid, the rate was faster and the same products were formed (results not shown). One may conclude that, in the presence of acid, **6b** is readily dehydrated to **2b**, which can then add ethanol to form **4b** (Scheme 1). Alternatively, **2b** can add water to form **5b** (16), which may react with ethanol in acid solution to form **4b**. Formation of **9b**, and particularly **1b**, in this reaction is more difficult to explain. Compound **9b** is believed to be formed by the oxidative deformylation of **5b** (12) (see Equation 4). However, as the reaction of **6b** with sulfuric acid was performed under nitrogen, the only oxidant present was **6b** itself. A possible explanation is that **6b** was dehydrated by acid to **2b**, which then added water to form **5b**. Compound **5b** is a phenol (reducing agent) that may be able to react with **6b** (oxidizing agent) to form **9b** (oxidation product) and the hydroquinone of **6b** (**6bH$_2$**; reduction product). In acid solution, **6bH$_2$** would cyclize to **1b** (11). To test this hypothesis, compound **6b** was reacted with **5b** at 60°C in the presence of 0.001 M sulfuric acid (to simulate the approximate pH of the **1b** oxidation) (Fig. 4). Compound **4b** was the major product, and **9b**, **7b** and **1b** were formed in smaller amounts. Although initially there were equal amounts of **6b** and **5b** in the reaction mixture, it appears that the first step involves the effective isomerization of **6b** into more **5b**, and thus the concentration of **5b** rises as that of **6b** falls.

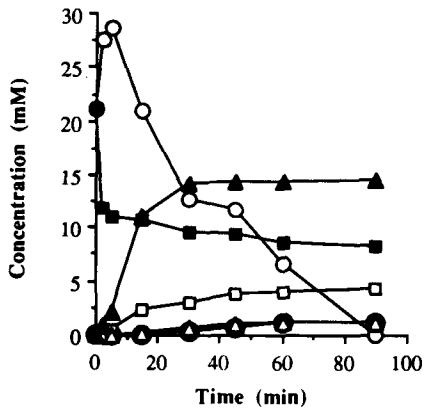


FIG. 4. Formation of products in the reaction of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b) with 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5b) in the presence of 0.001 M sulfuric acid in absolute ethanol at 60°C under nitrogen; □, 2,2,5,7,8-pentamethyl-6-chromanol (1b); ▲, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b); ○, 5b; ■, 6b; ●, 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (7b); △, 2,2,7,8-tetramethylchroman-5,6-dione (9b).

$$\mathbf{6b} \xrightarrow{H^+,\ -H_2O,\ +H_2O} \mathbf{5b} \qquad [2]$$

A number of reactions then follow. Compound **5b** reacts very readily with ethanol in the presence of acid to form **4b** (Eq. 3; Fig. 5), and the reaction does not require **6b**.

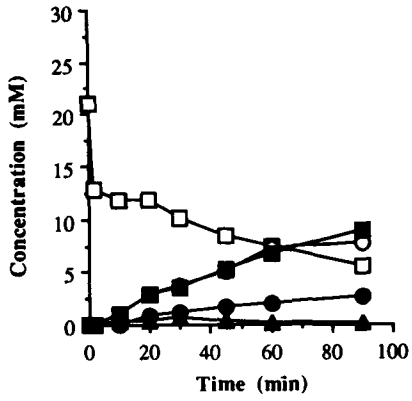


FIG. 3. Formation of products in the reaction of 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b) with 0.03 M sulfuric acid in absolute ethanol at 60°C under nitrogen; ■, 2,2,5,7,8-pentamethyl-6-chromanol (1b); ○, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b); □, 6b; ▲, 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (7b); ●, 2,2,7,8-tetramethylchroman-5,6-dione (9b).

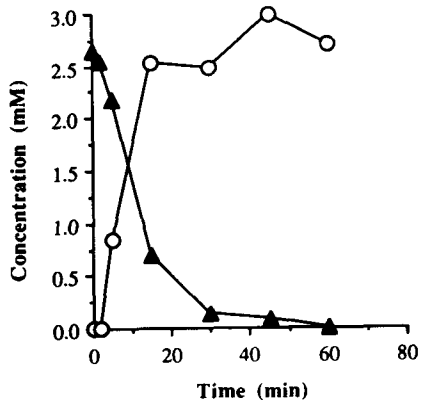


FIG. 5. Formation of 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b) from the reaction of 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5b) in the presence of 0.001 M sulfuric acid in absolute ethanol at 60°C; ○, 4b; ▲, 5b.

$$5b + EtOH \xrightarrow{H^+} 4b \quad [3]$$

Compound **6b** reacts with **5b** in the presence of acid (but not in its absence) to form **9b** and **1b** (Fig. 4). The conversion of **5b** into **9b** is a four-electron oxidation, and, therefore, the production of one mole of **9b** would require two additional moles of **6b** and lead to the formation of two moles of **1b** (Eqs. 4 and 5)

$$2 \times 6b + 5b + H_2O \xrightarrow{H^+} 2 \times 6bH_2 + 9b + HCHO \quad [4]$$

$$2 \times 6bH_2 \xrightarrow{2H^+} 2 \times 1b + 2H_2O \quad [5]$$

In summary, Equations 2, 4 and 5 give:

$$3 \times 6b \rightarrow 9b + 2 \times 1b + H_2O + HCHO \quad [6]$$

It is possible to conceive of an additional pathway from **6b** to **9b**, which does not involve the intermediacy of **5b** (Scheme 2). Thus, enolization of **6b**, followed by the addition of water, would yield 1,4-dihydroxy-2,3-dimethyl-5-(3-hydroxy-3-methylbutyl)-6-hydroxymethylbenzene (**8bH$_2$**), the hydroquinone of **8b**.

$$6b + H_2O \rightarrow 8bH_2 \quad [7]$$

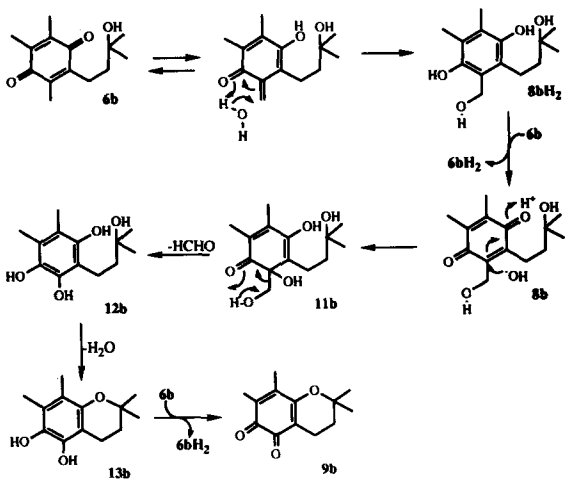
A second molecule of **6b** could then oxidize **8bH$_2$** to **8b** and be reduced to **6bH$_2$**:

$$6b + 8bH_2 \rightarrow 8b + 6bH_2 \quad [8]$$

Addition of water to **8b** would form 4,6-dihydroxy-2,3-dimethyl-5-(3-hydroxy-3-methylbutyl)-6-hydroxymethylcyclohexa-2,4-dienone (**11b**), which could deformylate to 1,2,4-trihydroxy-5,6-dimethyl-3-(3-hydroxy-3-methylbutyl)benzene (**12b**).

$$8b + H_2O \rightarrow 11b \quad [9]$$

$$11b \rightarrow 12b + HCHO \quad [10]$$



SCHEME 2

As discussed previously (12), the deformylation is a typical retro-aldol type of reaction whose driving force is the establishment of aromaticity in **12b**. Cyclization of **12b** would produce 2,2,7,8-tetramethyl-5,6-dihydroxychroman (**13b**), which could be oxidized by a third molecule of **6b** to form **9b**.

$$12b \rightarrow 13b + H_2O \quad [11]$$

$$6b + 13b \rightarrow 9b + 6bH_2 \quad [12]$$

Cyclization of **6bH$_2$** in acid conditions would yield **1b**:

$$2 \times 6bH_2 \rightarrow 2 \times 1b + 2H_2O \quad [13]$$

The sum of Equations 7–13 is Equation 6.

In order to determine whether **4b** could be converted into **9b**, it was oxidized with silver nitrate. Figure 6 shows that oxidation of **4b**, although relatively rapid, is much slower than that of **1b** and appears to go through a 20-min lag period before commencing. During the lag period the pH dropped from 6.2 to 4.2 (insert, Fig. 6), so that acid appears to play a role in this reaction also. Compound **9b** was a major product of the reaction, but it did not appear until after 30 min and was, therefore, a secondary product. The first-formed product was again compound **6b**, which reached a maximum in 90 min and then began to decline. Thus it appeared that **4b** was being converted *via* **2b** into **6b** and then into **9b** as before. Although compound **9b** formed more slowly than **6b**, it reached a higher concentration at 240 min and was formed more rapidly than in the oxidation of **1b**.

Compound **5b**, which was believed to be a key intermediate in the formation of **9b** from **1b** (12), was oxidized completely by silver nitrate within 20 min (Fig. 7). Although **9b** was the major product of the reaction (90%

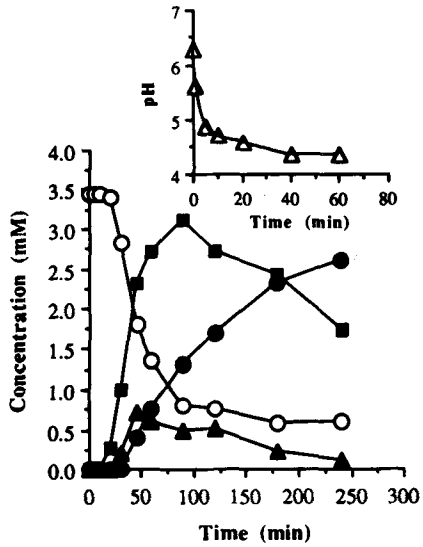


FIG. 6. Formation of products in the reaction between silver nitrate and 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b) in absolute ethanol at 60°C; ○, 4b; ■, 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6b); ▲, 2-(3-hydroxy-3-methylbutyl)-3-ethoxymethyl-5,6-dimethyl-1,4-benzoquinone (7b); ●, 2,2,7,8-tetramethylchroman-5,6-dione (9b). Insert shows the change in pH during the course of reaction.

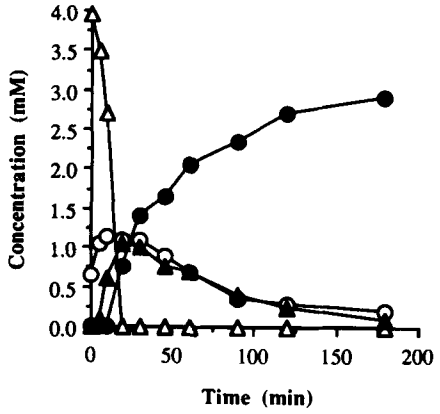


FIG. 7. Formation of products in the reaction between silver nitrate and 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5b) in absolute ethanol at 60°C; ○, 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4b); △, 5b; ▲, 2-(3-hydroxy-3-methylbutyl)-3-hydroxymethyl-5,6-dimethyl-1,4-benzoquinone (8b); ●, 2,2,7,8-tetramethylchroman-5,6-dione (9b).

after 180 min) and was formed faster in this reaction than in any other reaction, it was formed predominantly after **5b** had disappeared. Thus it must be concluded that **9b** was, at least in part, a secondary product of this reaction as well. Compounds **4b** and **8b** were formed more rapidly than **9b** early in the reaction and subsequently declined. Thus, it would appear that **5b** undergoes three separate reactions simultaneously, oxidative deformylation to **9b**, oxidation to **8b** and reaction with ethanol to form **4b**. Again it appeared that **4b** and **8b** were being converted into **9b**. A possible pathway from **4b** to **9b** would be protonation of the ethoxy oxygen of **4b**, followed by loss of ethanol to form the quinone methide **2b**. Addition of water to **2b** would form **5b**, oxidative deformylation of which would yield **9b**. The pathway by which **8b** is converted into **9b** is not clear but may involve acid-catalyzed cyclization to the enol of 5-formyl-2,2,7,8-tetramethyl-6-chromanol followed by oxidative loss of formic acid to form **9b**.

DISCUSSION

Compound **9b** can be formed from **1b**, **4b**, **5b** and **6b** and is formed most rapidly from **5b**, suggesting that **5b** is the immediate precursor of **9b**. In all oxidations except that of **6b**, compound **9b** was a secondary product. Conversion of **1b** into **9b** may be explained as in Scheme 1. Reaction of silver ion with **1b** produces silver metal and the radical cation of **1b** (Eq. 1). Dissociation of the radical cation liberates the chromanoxyl radical of **1b** and a proton which causes an immediate drop in pH (Eq. 1). Two molecules of the chromanoxyl radical disproportionate to give one molecule of the unstable intermediate quinone methide **2b** and one molecule of **1b** (21-23). Compound **2b** can react immediately with ethanol to form **4b**. Alternatively, **2b** may react with a proton to form **3b**, which can then react with water to form **6b** and regenerate a proton. Compound **6b** is unstable in the presence of acid and undergoes dehydration and recyclization into **2b**. Compound **2b** then undergoes hydration to **5b** and oxidative deformylation to **9b**. Although silver nitrate is the oxidant for the deformylation of **5b**, it is interesting to note that **6b** is also capable of bringing about the deformylation of **5b** with the concomitant formation of **1b** in mild acid conditions. It is also possible that **6b** may be converted into **9b** without the intermediacy of **5b** as shown in Scheme 2.

Acid also promotes the loss of ethanol from **4b** and its conversion into **2b**. Surprisingly, in this reaction **2b** does not proceed directly to **5b** and **9b** but rather to **6b**. Then as the acid increases further, **6b** is recyclized to **2b** and then to **5b** and **9b**. Thus the oxidation of **1b** is under the control of the equilibria **4b** ↔ **2b** ↔ **3b** ↔ **6b** (Scheme 1). In the absence of water, the major product is **4b** (20). In the presence of water, the equilibria shift to the right and a mixture of **4b** and **6b** will be formed, with **6b** dominating as the concentration of water rises. Then as the acid increases, the equilibria shift to the left and to the key intermediate **2b**, which undergoes hydration and further oxidation.

It is worth noting that the spirodimer and spirotrimer of **1b** were not observed in any of the reactions. This confirms previous observations that **2b** shows a much greater tendency to react with nucleophiles, such as water and alcohols, than it does to polymerize (20).

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Synthesis and Antitumor Activity of 1-β-D-Arabinofuranosylcytosine Conjugates of Optical Isomers of Ether and Thioether Lipids[1]

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Four 1-β-D-arabinofuranosylcytosine conjugates (ara-C) (1a, b and 2a, b) of *sn*-1 and *sn*-3 isomers of 1-*O*-octadecyl-2-*O*-palmitoylglycerol and its 1-*S*-alkyl analogue have been synthesized, and their antitumor activity against L1210 lymphoid leukemia in mice were compared with those of the previous conjugates (3a, b) of racemates in order to determine the significance of chirality of the glycerol moieties for activity. Administration (i.p.) of a single dose (300 mg/kg) of conjugates of *sn*-1 (1a), *sn*-3 (2a) and *rac* (3a) isomers of the ether lipid increased lifespan of i.p. implanted L1210 lymphoid leukemic DBA/2J mice by 169, 175 and 236%, respectively. The *sn*-1 (1b), *sn*-3 (2b), and *rac* (3b) isomers of the thioether lipid with a single dose of 300 mg/kg produced an increase in lifespan values of 238, 263 and 250%, respectively. The results indicate that chirality of the glycerol moieties appears not to be critical for the activity, and racemates 3a and 3b are promising prodrugs of ara-C for further clinical investigations.
Lipids 28, 1021-1026 (1993).

1-β-D-Arabinofuranosylcytosine (ara-C) conjugates of the racemic mixtures of 1-*O*-alkyl (ether) and 1-*S*-alkyl (thioether) phospholipids (1-10) have demonstrated a superior antitumor activity against animal tumor models *in vivo*. The rationale is that the conjugates are not only new prodrugs of ara-C, but also may generate two cytotoxic groups, the nucleoside and the ether or thioether lipid analogue, with different target sites in tumor cells. Among them, ara-CDP-*rac*-1-*S*-octa-decyl-2-*O*-palmitoyl-1-thioglycerol (ara-CDP-DL-PTBA, Cytoros, **3b**) (Fig. 1) showed significant therapeutic effects on human colorectal (11) and PSN-1 pancreatic cancer xenografts in nude mice (12). In order to determine the stereochemical significance of the ether and thioether moieties, we have now synthesized the ara-C conjugates of the *sn*-1 (**1a, b**) and *sn*-3 (**2a, b**) isomers of 1-*O*-octadecyl-2-*O*-palmitoylglycerol and its 1-thioglycerol isomer (Fig. 1). This paper described the synthetic procedures for the preparation of these conjugates and the comparison of their antitumor effects with those of the previous conjugates (**3a** and **3b**) of the racemates of ether and thioether lipids against L1210 lymphoid leukemia in mice.

EXPERIMENTAL PROCEDURES

Melting points were taken on a Mel-Temp capillary melting point apparatus (Cambridge, MA) and are uncorrected. ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associate (Sunnyvale, CA) EM-390 spectrometer. Chemical shifts are expressed as δ values (ppm) relative to tetramethylsilane as internal standard. The optical rotations were measured on a Perkin-Elmer 241 polarimeter (Norwalk, CT). An AG1-X8 (Bio-Rad, Hercules, CA), [(diethylamino)ethyl]cellulose (DE-52) (Whatman, Clifton, NJ) and a CG-50 (Sigma, St. Louis, MO) were used for column chromatography. Evaporations were carried out on a rotary evaporator under reduced pressure applied by an Aspirator A-3S (Wheaton, Millville, NJ) or a vacuum pump with a bath temperature of under 30°C. Thin-layer chromatography (TLC) was performed on glass plates coated with a 0.25-mm layer of silica gel PF-254 (Brinkman, Westbury, NY) with use of the following solvent systems: (A) $CHCl_3$, (B) $CHCl_3$/MeOH (95:5, vol/vol), (C) $CHCl_3$/MeOH/H_2O/HOAc (25:15:4:2, by vol), and *i*-PrOH/H_2O/concentrated NH_4OH (7:2:1, by vol). Ultraviolet (UV)-absorbing compounds were detected by visualization under a UV lamp (254 nm), and phosphorus-containing compounds were detected with a modified Dittmer-Lester spray (13). Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN) and Robertson Microlit Laboratories (Madison, NJ).

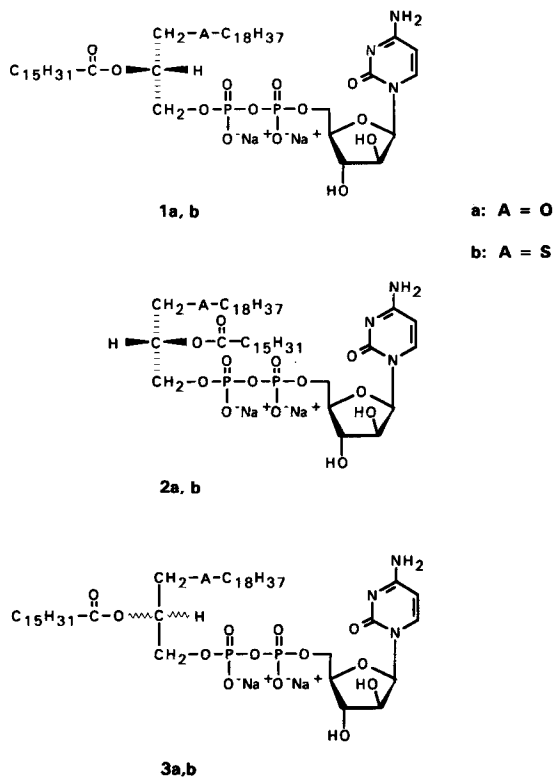

FIG. 1. Structures of 1-β-D-arabinofuranosylcytosine conjugates of 1-*O*-(or *S*)-octadecyl-2-*O*-palmitoylglycerol (or 1-thioglycerol).

[1]This material was presented in part at the 81st Annual Meeting of the American Association for Cancer Research in Washington, D.C., May, 1990 (Abstract No. 2493).

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Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CDP, ara-C 5′-diphosphate; ara-CDP-DL-PTBA (Cytoros), ara-CDP-*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol; ara-CMP, ara-C 5′-monophosphate; ILS, increase in life span; NMR, nuclear magnetic resonance; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet.

Ara-C 5′-monophosphate (ara-CMP) (14), ara-CMP morpholidate (15), 1,2-*O*-isopropylidene-*sn*-glycerol (16), 2,3-*O*-isopropylidene-*sn*-glycerol (17), 1,2-*O*-isopropylidene-3-thio-*sn*-glycerol (18) and racemic compounds **3a, 3b, 6a, 6b, 9a** and **9b**) were prepared by procedures in the literature (1,7) .

2,3-*O*-Isopropylidene-1-thio-*sn*-glycerol was prepared from 2,3-*O*-isopropylidene-*sn*-glycerol in an analogous manner for 1,2-*O*-isopropylidene-3-thio-*sn*-glycerol (18), and was used for the next step without distillation.

1-O-Octadecyl-sn-*glycerol* and *3-O-Octadecyl*-sn-*glycerol.* These compounds were prepared following literature procedures (19,20).

1-O-Octadecyl-2-O-palmitoyl-sn-*glycerol (4a).* Palmitoyl chloride (6.95 g, 25 mmol) in toluene (20 mL) was added dropwise to a mixture of 1-*O*-octadecyl-3-*O*-benzyl-*sn*-glycerol (21) (10 g, 23 mmol), pyridine (1.76 g, 23 mmol) and toluene (50 mL) at room temperature. The mixture was heated at 60°C overnight and then cooled to room temperature. The mixture was partitioned between Et_2O (150 mL) and H_2O (150 mL), and the organic layer was washed with H_2O (2 × 150 mL) and evaporated to dryness. The residue was dissolved in boiling 95% EtOH (1500 mL), and the solution was cooled to room temperature overnight. The solid, 1-*O*-octadecyl-2-*O*-palmitoyl-3-*O*-benzyl-*sn*-glycerol, was filtered, washed with cold 95% EtOH and dried *in vacuo*; yield, 13.1 g (84.6%); m.p., 43–44.5°C; $[\alpha]_D^{25}$ +1.4° (c 8.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.9 (6, *t*, J = 6 Hz, 2 CH_3), 1.18–1.74 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.35 (2, *t*, J = 6 Hz, CH_2CO), 3.44 (2, *t*, J = 6 Hz, OCH_2), 3.56 (2, *d*, J = 5 Hz, 1-CH_2), 3.63 (2, *d*, J = 5 Hz, 3-CH_2), 4.52 (2, *s*, benzyl CH_2), 5.16 (1, *quintet*, J = 5 Hz, 2-CH), 7.26 (5,*s*, C_6H_5). The compound (12.5 g, 18.6 mmol) was hydrogenated with 10% Pd/C (1 g) in tetrahydrofuran (THF) (100 mL) and HOAc (50 mL) at 50 psi for three days. After removal of the catalyst, the filtrate was evaporated to dryness, and the residue was crystallized from $CHCl_3$-petroleum ether 30–60°C; yield, 9.8 g (74.9%); m.p., 70–71°C; $[\alpha]_D^{25}$ −0.15° (c 8.0, $CHCl_3$). *Anal.*: Calcd. for $C_{37}H_{74}O_4$: C, 76.23; H.12.79. Found: C, 76.00; H.12.76.

3-O-Octadecyl-2-O-palmitoyl-sn-*glycerol (5a).* 3-*O*-Octadecyl-2-*O*-palmitoyl-1-*O*-trityl-*sn*-glycerol (17.7 g, 21.4 mmol), prepared by the procedure in the literature (1), was dissolved in HOAc (200 mL) and toluene (50 mL) and hydrogenated in the presence of PtO_2 (500 mg) and PdO (700 mg) at 50 psi for five days. The catalysts were removed by filtration, the filtrate was evaporated to dryness and the residue was crystallized from 95% EtOH/hexanes (5:1, vol/vol); yield, 10.5 g (84.2%); m.p., 51–52°C; $[\alpha]_D^{22}$ +2.0° (c 1, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.85 (6, *t*, J = 5 Hz, 2 CH_3), 1.27–1.65 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.30 (2, *m*, CH_2CO), 3.42 (2, *t*, J = 6 Hz, OCH_2), 3.58 (2, *d*, J = 5 Hz, 1-CH_2), 3.77 (2, *d*, J = 5 Hz, 3-CH_2), 4.97 (1, *quintet*, J = 5 Hz, 2-CH). The product was also prepared in 46% yield by using the method for **4b**. *Anal.*: Calcd. for $C_{37}H_{74}O_4$: C, 76.23; H, 12.79. Found: C, 76.26; H, 12.33.

3-S-Octadecyl-3-thio-sn-*glycerol.* Two hundred mL of 1 N KOH in MeOH was added dropwise to a mixture of 1,2-*O*-isopropylidene-3-thio-*sn*-glycerol (29.6 g, 0.2 mol) in 100 mL of MeOH and 1-bromooctadecane (60.1 g, 0.18 mol) in 200 mL of hexanes, at room temperature for a period of 1 h, and then the mixture was stirred at room temperature for 1 d. The mixture was evaporated to dryness, and the residue was partitioned between hexanes (800 mL) and H_2O (300 mL). The organic layer was evaporated to dryness, and the residue was refluxed with 10% HCl in aqueous MeOH (125 mL concentrated HCl and 280 mL MeOH) for 30 min and decolorized with Norit A (Fisher Scientific, Pittsburgh, PA). After being left to stand at room temperature overnight, the solid in the mixture was filtered off, washed with 50% MeOH and then MeOH (50 mL each), and then dried *in vacuo.* The crude product was recrystallized from hot MeOH; yield, 49.2 g (75.8%); m.p., 67–68°C; $[\alpha]_D^{22}$ +22.1° (c 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.83 (3, *t*, J = 6 Hz, CH_3), 1.27–1.65 (32, *m*, $(CH_2)_{16}$), 2.40–2.63 (4, *m*, CH_2SCH_2), 3.33 (2, *t*, J = 6 Hz, 1-CH_2), 3.63 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{21}H_{44}O_2S{\cdot}0.1H_2O$: C, 69.59; H, 12.26. Found: C, 69.49; H, 12.48.

1-*S*-Octadecyl-1-thio-*sn*-glycerol was prepared from 2,3-*O*-isopropylidene-1-thio-*sn*-glycerol in an analogous manner; yield, 52.9%; m.p., 66–67°C; $[\alpha]_D^{22}$ +9.0° (c 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.87 (3, *t*, J = 6 Hz, CH_3), 1.27–1.57 (32, *m*, $(CH_2)_{16}$), 2.43–2.68 (4, *m*, CH_2SCH_2), 3.35 (2, *t*, J = 6 Hz, 3-CH_2), 3.77 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{21}H_{44}O_2S{\cdot}0.75H_2O$: C, 67.42; H, 12.26. Found: C, 67.25; H, 11.63.

1-S-Octadecyl-3-O-(tert-butyldimethylsilyl)-1-thio-sn-*glycerol.* A mixture of 1-*S*-octadecyl-1-thio-*sn*-glycerol (9.15 g, 25 mmol), *tert*-butyldimethylsilyl chloride (4.66 g, 30 mmol), imidazole (4.13 g, 60 mmol) and *N,N*-dimethylformamide (DMF) (50 mL) was stirred at room temperature for one day. The solvent was evaporated to dryness *in vacuo* at 70°C, and the residue was partitioned between H_2O and Et_2O (50 mL each). The organic layer was dried over Na_2SO_4 and then evaporated to dryness. The oily residue was further evaporated by using a high vacuum at 70°C. The crude product, essentially homogeneous by TLC, weighed 10.4 g (87.6%) and was used for the next step without further purification.

1-*O*-(*tert*-Butyldimethylsilyl)-3-*S*-octadecyl-3-thio-*sn*-glycerol was prepared by using an analogous procedure; yield, 85.7%.

1-S-Octadecyl-2-O-palmitoyl-1-thio-sn-*glycerol (4b).* Palmitoyl chloride (7.01 g, 25 mmol) was added dropwise to a mixture of the above product (10.4 g, 22 mmol) in anhydrous pyridine (10 mL) and toluene (400 mL) at room temperature, and the mixture was stirred at room temperature for one day. The mixture was then partitioned between Et_2O and H_2O (100 mL each). The organic layer was washed with 0.5 N H_2SO_4, saturated $NaHCO_3$ and H_2O (50 mL each), and then evaporated to dryness. The residue was crystallized from 95% EtOH at 10–15°C. The solid was filtered and washed with 95% EtOH. The soft solid, 1-*S*-octadecyl-2-*O*-palmitoyl-3-*O*-(*tert*-butyldimethylsilyl)-1-thio-*sn*-glycerol, weighed 13.0 g (81.8% yield). To a mixture of this product (13.0 g, 18 mmol) in HOAc (3.5 mL) and THF (100 mL) was added dropwise 1 M tetrabutylammonium fluoride in THF (35 mL) for a period of 1 h at 5–10°C, and the mixture was stirred at room temperature for 6 h. After cooling at 0–5°C overnight, the solid was filtered and washed with ice-cold 95% EtOH. The filtrate was evaporated to dryness, and the residue was treated with ice-cold 95% EtOH. The combined solids were dissolved in boiling 95% EtOH, and the solution was cooled to room temperature overnight, which resulted in an isomer of **4b**, 1-*S*-octadecyl-3-*O*-palmitoyl-1-thio-*sn*-

glycerol. After filtration, the filtrate was cooled at 0–5°C overnight, and the white solid (**4b**) was filtered and washed with cold 95% EtOH. Repeated recrystallization of the crude products in this manner gave 6.7 g (50.0% yield); m.p., 44–45°C; $[\alpha]_D^{22}$ +0.6° (c 1.0, $CHCl_3$); ^1H NMR δ 0.87 (6, *m*, $2CH_3$), 1.27–1.60 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.32 (2, *t*, J = 7 Hz CH_2CO), 2.47–2.73 (4, *m*, CH_2SCH_2), 3.80 (2, *m*, 3-CH_2), 4.97 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{37}H_{74}O_3S$: C, 74.18; H, 12.45. Found: C, 74.24; 12.78.

3-*S*-Octadecyl-2-*O*-palmitoyl-3-thio-*sn*-glycerol (**5b**) was prepared in an analogous manner; yield, 28.8%; m.p., 45–46°C; $[\alpha]_D^{22}$ +1.0° (c 1.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 4.93 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{37}H_{74}O_3S$: C, 74.18; H, 12.45. Found: C, 73.99; H, 11.79.

3-O-Octadecyl-2-O-palmitoyl-sn-*glycerol-1-phosphate (8a).* Phenyl dichlorophosphate (1.45 g, 6.8 mmol) in toluene (30 mL) was added dropwise at room temperature to a solution of **4b** (2.0 g, 3.4 mmol), pyridine (1 mL) and toluene (30 mL), and the mixture was stirred first at 60°C for 6 h and then at room temperature for 1 d. Water (1 mL) was added to the mixture, and the suspension was stirred at room temperature for 1 h. The mixture was then partitioned between Et_2O and H_2O (40 mL each). The organic layer was washed with H_2O (40 mL × 3), dried over Na_2SO_4 and evaporated to dryness. The residue was crystallized from 95% EtOH at −20°C, and the solid was quickly filtered and washed with cold 95% EtOH; yield, 2.0 g (79.6%). The phenyl-protected phosphate was then hydrogenated with PtO_2 (400 mg) and PdO (400 mg) in AcOH (50 mL) and toluene (50 mL) at 50 psi for 2 d. The mixture was diluted with toluene (30 mL) and filtered through a Celite bed. The filtrate was evaporated to dryness, and the residue was dried by coevaporating with toluene (10 mL × 2). The crude product was dissolved in $CHCl_3$ (20 mL), and the solution was applied to a silica gel column (3 × 30 cm). The column was eluted with $CHCl_3$ (400 mL) and then with $CHCl_3$/MeOH/H_2O (10:10:1, by vol) (250 mL). The eluate with the latter was evaporated to dryness and the residue weighed 1.8 g (100%, 79.6% overall); m.p., 49–51°C; $[\alpha]_D^{22}$ +5.55° (c 1.0, $CHCl_3$); ^1H NMR δ 0.83 (6, *m*, $2CH_3$), 1.23–1.56 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.26 (2, *t*, J = 6 Hz, CH_2CO), 3.33–3.43 (4, *m*, OCH_2, 3-CH_2), 4.08 (2, *d*, J = 4.5 Hz, 1-CH_2), 5.12 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{37}H_{75}O_7P{\cdot}4.5H_2O$: C, 59.72; H, 10.56. Found: C, 59.37; H, 11.38.

1-S-Octadecyl-2-O-palmitoyl-1-thio-sn-*glycerol-3-phosphate (7b).* Triethylamine (7.08 g, 70 mmol) in hexanes (20 mL) was added dropwise to an ice-cold mixture of $POCl_3$ (9.96 g, 70 mmol) and hexanes (20 mL). To this mixture a solution of dried **4b** (32.1 g, 50 mmol) in toluene (250 mL) was added dropwise at 0–5°C over a period of 1.5 h, and the mixture was stirred at room temperature overnight. Water (25 mL) was added to the mixture followed by stirring at room temperature for 1 h. The mixture was partitioned between Et_2O (250 mL) and H_2O (125 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was crystallized from hexanes at 0–5°C and then recrystallized from Et_2O at room temperature; yield, 21.4 g (45%); m.p., 65–70°C; $[\alpha]_D^{22}$ +3.6° (c 1.0, $CHCl_3$); ^1H NMR δ 0.85 (6, *t*, J = 7 Hz, $2CH_3$), 1.27–1.53 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.20–2.73 (6, *m*, CH_2CO, CH_2SCH_2), 4.03 (2, *m*, 3-CH_2), 5.03 (1, *m*, 2-CH). *Anal.*: Calcd. for $C_{37}H_{75}O_6SP{\cdot}0.5H_2O$: C, 64.59; H, 11.13. Found: C, 64.26; H, 11.34. Phosphatidic acids **7a** and **8b** were prepared in an analogous manner.

1-O-Octadecyl-2-O-palmitoyl-sn-*glycerol-3-phosphate (7a).* Yield, 74.0%; m.p., 58–60°C; $[\alpha]_D^{22}$ +1.1° (c, 3.55, $CHCl_3$). *Anal.*: Calcd. for $C_{37}H_{75}O_7P$: C, 67.03; H, 11.40; P, 4.67. Found: C, 66.83; H, 11.45; P, 4.32.

3-S-Octadecyl-2-O-palmitoyl-3-thio-sn-*glycerol 1-phosphate (8b).* Yield, 50.0%; m.p., 60–70°C; $[\alpha]_D^{22}$ +1.5° (c 1.0, $CHCl_3$); ^1H NMR 5.03 (1, *m*, 2-CH). The product was used for the condensation with ara-CMP morpholidate without chemical analysis.

ara-C-5-diphosphate-(ara-CDP)-1-S-octadecyl-2-O-palmitoyl-1-thio-sn-*glycerol (1b).* Phosphate **7b** (3.40 g, 5 mmol) was dried azeotropically with pyridine twice and mixed with ara-CMP morpholidate (2.75 g, 4 mmol), followed by co-evaporation with pyridine three times. The dried mixture was then mixed with anhydrous pyridine (300 mL) and stirred at room temperature for five days. The solvent was evaporated to dryness, and the residue was co-evaporated with toluene to remove the residual pyridine. The residue was dissolved in 300 mL of $CHCl_3$/MeOH/H_2O (2:3:1, by vol) and then mixed with 1 N HCl (11 mL) and H_2O (26 mL). The organic layer was separated, and the aqueous layer was extracted with $CHCl_3$ (2 × 50 mL). The combined organic layers were evaporated to dryness, and the residue was dissolved in 400 mL of $CHCl_3$/MeOH/H_2O (2:3:1, by vol). The solution was applied to a DE-52 (AcO^-) column (5 × 30 cm) prepacked with the solvent. The column was eluted with $CHCl_3$/MeOH/H_2O (2:3:1, by vol) (1000 mL) and then with 0.04 M NH_4OAc in the same solvent. The 0.04 M NH_4OAc fractions between 2100–4700 mL were evaporated to a small volume, and the solid was collected on a filter, followed by washing with 50% aqueous Me_2CO and then Me_2CO. The solid (NH_4 salt of 1b) was dissolved in $CHCl_3$/MeOH/H_2O (2:3:1, by vol), and the solution was passed through a CG-50 (Na^+) column (2 × 10 cm). The column was washed further with the same solvent until no UV-absorbing material was detected. The combined eluate was cooled at 0–5°C overnight, and the solid was filtered off. The filtrate was evaporated to a small volume and the product (Na salt) was filtered, washed with acetone and dried *in vacuo*; yield, 2.44 g (59.3%); m.p., 200–201°C; $[\alpha]_D^{22}$ +31.0° (c 1, $CHCl_3$-MeOH-H_2O 2:3:1, by vol); ^1H NMR ($CDCl_3$-CD_3OD-D_2O, 2:3:1, by vol) δ 0.83 (6, *t*, J = 6 Hz, $2CH_3$), 1.27–1.73 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.32 (2, *t*, J = 7 Hz, CH_2CO), 2.50 (2, t, J = 7 Hz, SCH_2), 2.76 (2, *t*, J = 7 Hz, 1-CH_2), 3.97–4.73 (7, *m*, 3-CH_2, H-2′, H-3′, H-4′, H-5′), 5.07 (1, *m*, 2-CH), 5.92 (1, *d*, J = 7.5 Hz, cytosine H-5), 6.08 (1, *d*, J = 5 Hz, H-1′), 7.80 (1, *d*, J = 7.5 Hz, cytosine H-6). *Anal.*: Calcd. for $C_{46}H_{85}N_3O_{13}SP_2{\cdot}2Na{\cdot}H_2O$: C, 52.81; H, 8.38; N, 4.01. Found: C, 52.97; H, 8.44; N, 3.61. The conjugates **1a**, **2a** and **2b** were prepared in an analogous manner.

ara-CDP1-O-octadecyl-2-O-palmitoyl-sn-*glycerol (1a).* Yield, 24.4%; m.p., 156–164°C; $[\alpha]_D^{22}$ +33.5° (c, 0.23, $CHCl_3$/MeOH/H_2O, 2:3:1, by vol); ^1H NMR ($CDCl_3$/CD_3OD/D_2O, 2:3:1, by vol) δ0.87 (6, *m*, $2CH_3$), 1.25–1.80 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.33 (2, *m*, CH_2CO), 3.36–4.13 (11, *m*, 1-CH_2, 3-CH_2, OCH_2, H-2′, H-3′, H-4′, H-5′), 4.97 (1, *m*, 2-CH), 5.92 (1, *d*, J = 7.5 Hz, cytosine H-5), 6.14 (1, *d*, J = 5 Hz, H-1′), 7.88 (1, *d*, J = 7.5 Hz, cytosine H-6). *Anal.*: Calcd. for $C_{46}H_{87}N_3O_{14}P_2 3CH_3OH$: C, 56.42; H, 9.06; N, 3.95; P, 5.82. Found: C, 56.82; H, 9.27; N, 3.69; P, 5.87.

ara-CDP3-O-octadecyl-2-O-palmitoyl-sn-glycerol (2a). Yield, 26.2%; m.p., 200–205°C; $[\alpha]_D^{22}$ +25.6° (c, 0.39, $CHCl_3$/MeOH/H_2O, 2:3:1, by vol); ^1H NMR ($CDCl_3$/CD_3OD/D_2O, 2:3:1, by vol) δ0.94 (6, *m*, $2CH_3$), 1.15–1.85 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.37 (2, *t*, *J* = 7 Hz, CH_2CO), 3.25–4.40 (11, *m*, 1-CH_2, 3-CH_2, OCH_2, H-2′, H-3′, H-4′, H-5′), 5.16 (1, *m*, 2-CH), 5.97 (1, *d*, *J* = 7.5 Hz, cytosine H-5), 6.16 (1, *d*, *J* = 5 Hz, H-1′), 7.82 (1, *d*, *J* = 7.5 Hz, cytosine H-6). *Anal.*: Calcd. for $C_{46}H_{85}N_3O_{14}P{\cdot}2Na{\cdot}0.5(CH_3)_2CO$: C, 55.05; H, 8.66; N, 4.01; P, 5.92. Found: C, 54.84; H, 9.16; N, 3.55; P, 6.12.

ara-CDP3-S-octadecyl-2-O-palmitoyl-3-thio-sn-glycerol (2b). Yield 20.4%; m.p., 198–200°C; $[\alpha]_D^{22}$ +18.4° (c 1.0, $CHCl_3$/MeOH/H_2O, 2:3:1, by vol); ^1H NMR ($CDCl_3$/CD_3OD/D_2O, 2:3:1, by vol) δ0.88 (6, *t*, *J* = 6 Hz, $2CH_3$), 1.24–1.71 (58, *m*, $(CH_2)_{16}$, $(CH_2)_{13}$), 2.38 (2, *t*, *J* = 7 Hz, CH_2CO), 2.59 (2, *t*, *J* = 7 Hz, SCH_2), 2.79 (2, *m*, 3-CH_2), 4.12–4.80 (7, *m*, 1-CH_2, H-2′, H-3′, H-4′, H-5′), 4.93 (1, *m*, 2-CH), 6.02 (1, *d*, *J* = 7.5 Hz, cytosine H-5), 6.21 (1, *d*, *J* = 5 Hz, H-1′), 7.94 (1, *d*, *J* = 7.5 Hz, cytosine H-6). *Anal.*: Calcd. for $C_{46}H_{85}N_3O_{13}SP_2{\cdot}2Na$: C, 53.73; H, 8.33; N, 4.09. Found: C, 54.07; H, 8.37; N, 3.54.

Antitumor activity in vivo. DBA/2J male mice in groups of 5–8 (wt: 20–29 g) were inoculated i.p. with 1 × 10^6 L1210 lymphoid leukemia cells (22), and a sonicated solution of the conjugates was given i.p. once daily a single or multiple injections, starting at day 1, as outlined previously (8). Survival time and animal weights were recorded and the % increase in life span (%ILS) was compared with the median survival of the untreated mice. The results are shown in Table 1.

RESULTS

The synthetic route used to prepare ara-C conjugates **1a, b** and **2a, b** was analogous to that of our earlier work in this series with conjugates **3a** (1) and **3b** (7). Optically pure 1,2-*O*-isopropylidene-*sn*-glycerol (16), 2,3-*O*-isopropylidene-*sn*-glycerol (17) and the respective *O*-alkylated products 1-*O*-octadecyl-*sn*-glycerol (19) and 3-*O*-octadecylglycerol (19) were prepared by literature procedures (20). The 1-thioglycerol analogue, 1,2-*O*-isopropylidene-3-thio-*sn*-glycerol (18), was prepared by the procedure in the literature and its optical isomer, 2,3-*O*-isopropylidene-1-thio-*sn*-glycerol, was prepared from 2,3-*O*-isopropylidene-*sn*-glycerol in an analogous manner. Alkylation and subsequent removal of the isopropylidene group of these 1-thioglycerol derivatives gave 1-*S*-octadecyl-1-thio-*sn*-glycerol and 3-*S*-octadecyl-3-thio-*sn*-glycerol. After protecting the 1- or 3-OH group of the *O*-octadecyl-*sn*-glycerols with a benzyl or trityl, the 2-OH was acylated with palmitoyl chloride in the presence of pyridine, and subsequent removal of the benzyl or trityl protective group by catalytic hydrogenolysis with 10% Pd/C or PtO/PdO gave **4a** and **5a** in 46–84% yield (Fig. 2). The benzyl or trityl group of the *sn*-1 (or *sn*-3) *O*-octadecyl-2-*O*-palmitoylglycerol was removed with minimal acyl migration to form 1-*O*-octadecyl-3-*O*-palmitoyl-*sn*-glycerol or 3-*O*-octadecyl-2-*O*-palmitoyl-*sn*-glycerol. The 1- or 3-OH group of the *S*-octadecyl-thio-*sn*-glycerols was protected with *tert*-butyldimethylsilyl (TBDMS) group, and the 2-OH was acylated with palmitoyl chloride in the presence of pyridine. The TBDMS group was removed by treatment with tetrabutylammonium fluoride in THF and HOAc at 5–10°C and then at room temperature (7), resulting in **4b** and **5b** in 29–50% yield. The acyl migration during the deblocking was also minimal. However, the acyl migration occurred even during the crystallization in 95% EtOH. Structure assignments of **4a, b** and **5a, b** were confirmed by ^1H NMR spectrometry. The *sn*-2 methine proton of the above compounds gave a first-order quintet at 4.93–5.16 ppm, as demonstrated previously (1,7).

TABLE 1

Antitumor Activity Against i.p. Implanted L1210 Lymphoid Leukemia in Mice[a]

Compound	Treatment schedule, qd	Dose, mg (μmol)/kg per day	Survival days			45-Day survivors
			Range	Median T/C[b]	% ILS[c]	
ara-C.HCl	1	55 (196)	8–9	9.0/8.0	13	0/5
		81 (290)	9–10	9.0/9.0	0	0/6
	1–5	230 (822)	14–15	15.0/7.5	100	0/6
1a	1	100 (98)	11–21	14.5/8.0	81	0/6
		200 (196)	17–22	19.5/8.0	144	0/6
		300 (294)	18–25	21.5/8.0	169	0/6
2a	1	100 (98)	15–17	16.0/8.0	100	0/6
		200 (196)	16–23	21.0/8.0	163	0/6
		300 (294)	15–>45	22.0/8.0	175	1/6
3a	1	100 (98)	15–18	18.0/7.0	157	0/6
		200 (196)	19–>45	27.0/7.0	286	1/8
		300 (294)	21–28	23.5/7.0	236	0/6
1b	1	300 (292)	17–>45	27.0/8.0	238	2/5
	1,5,9	100 (97)	23–44	28.0/8.0	250	0/5
	1–5	60 (58)	22–>45	25.0/8.0	213	1/5
2b	1	300 (292)	23–>45	29.0/8.0	263	2/5
	1,5,9	100 (98)	8–25	22.0/8.0	175	0/5
	1–5	60 (58)	15–30	24.0/8.0	200	0/5
3b	1	300 (292)	21–>45	28.0/8.0	250	1/5
	1,5,9	100 (98)	18–29	25.0/8.0	213	0/5
	1–5	60 (58)	27–>45	31.0/8.0	288	1/5

[a]Each group of 5–8 DBA/2J mice (male, 20–29 g) received i.p. inoculation of 1 × 10^6 cells on day 0. Treatments (i.p.) were initiated on day 1. Ara-C, 1-β-D-arabinofuranosylcytosine.
[b]Calculated based on survivors according to the NCI protocols (22). T/C, treated/control (untreated).
[c]Increase in life span: (T/C − 1) × 100.

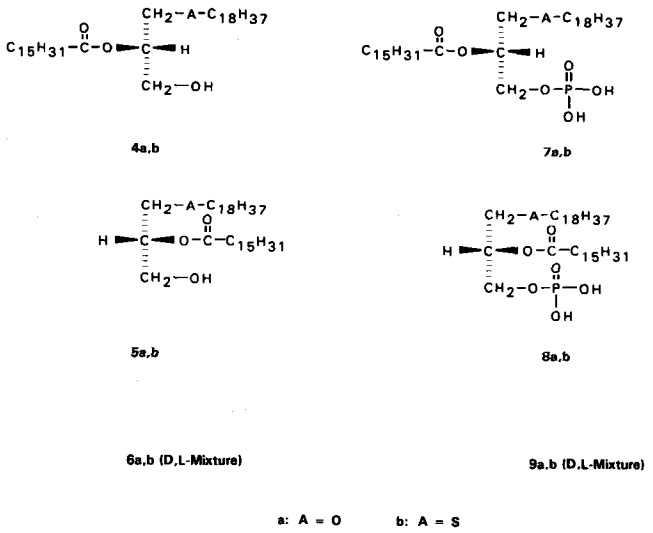

FIG. 2. Structures of ether and thioether lipids.

Compounds **4a, b** and **5a, b** were phosphorylated with $POCl_3$ and Et_3N at 0–5°C, as outlined previously (1), and the resulting phosphates, **7a, b** and **8a, b**, were purified by successive crystallization from hexanes and Et_2O, to obtain a final yield of 45–74%. Phosphate **8a** was also prepared in 80% yield by phosphorylation of **5a** with phenyl dichlorophosphate in the presence of pyridine and subsequent removal of the phenyl group by catalytic hydrogenolysis with PtO_2/PdO. Condensation of **7a, b** and **8a, b** with ara-CMP morpholidate (15) in pyridine gave conjugates **1a, b** and **2a, b** in overall yields of 20–59%, and their structures were verified by elemental analysis and ^1H NMR spectroscopy.

Antitumor activity. Conjugates **1a–3a** and **1b–3b** were compared for their *in vivo* antitumor activity against i.p. implanted L1210 lymphoid leukemia in DBA/2J mice, according to the procedures outlined in the NCI protocols (22), with some modifications, such as inoculation of 1×10^6 cells and 45-day observation. In Table 1, the untreated animals died on days 6–8 after tumor implantation. Conjugates of *sn*-1 (**1a**) and *sn*-3 (**2a**) isomers of the ether lipid injected i.p. as a single dose produced comparable ILS values among the isomers at each of three dose levels: 81 *vs.* 100% at 100 mg (98 μmol)/kg, 144 *vs.* 163% at 200 mg (196 μmol) and 169 *vs.* 175% at 300 mg (294 μmol)/kg on day 1. The racemic mixture (**3a**) at the same doses gave somewhat higher ILS values (157–286%) than the *sn*-1 and *sn*-3 isomers. However, a single i.p. injection of equimolar doses (196 and 290 μmol/kg) of ara-C failed to increase survival of the leukemic mice. Multiple high doses of ara-C (230 mg, 822 μmol/kg per day × 5) produced 100% ILS. Administration (i.p.) of a single dose (300 mg, 292 μmol/kg) of conjugates of *sn*-1 (**1b**), *sn*-3 (**2b**), and racemic mixture (**3b**) of the thioether lipid produced ILS values of 238, 263 and 250%, respectively, with 1–2 long-term survivors (<45 d) out of five animals. When a total 300 mg/kg of the conjugates was administered i.p. in three or five divided doses, the ILS values obtained were 175–288%.

DISCUSSION

Results obtained in this study demonstrated again that ara-C conjugates of ether and thioether lipids are highly effective against L1210 lymphoid leukemia in mice regardless of chirality at the glycerol, exceeding the activity of ara-C. Both racemic mixtures **3a** and **3b** showed a comparable activity to the *sn*-1 isomers **1a** and **1b**, as previously reported for the *sn*-1 isomer and racemic mixture of ara-CDP-dipalmitin (23). These conjugates contain both ara-C and an ether or thioether lipid in place of the cytidine and diacylglycerol, which are present in natural cytidine diphosphate diglyceride, a precursor for membrane phosphatidylinositol and cardiolipin (24). Thus, the conjugates may produce two cytotoxic groups, ara-C 5′-triphosphate (ara-CTP) and alkyllysophospholipid or 1-*S*-thioether lysophospholipid after biotransformation (1,8). Synthetic analogues of the latter lipids are membrane-active antitumor agents (25,26) and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and 1-hexadecylmercapto-2-methoxymethyl-*rac*-propane-3-phosphocholine (BM 41.440, Ilmofosine) are in clinical trials (27,28). These compounds are also racemic mixture. Thus, conjugates **3a** and **3b** (Cytoros) of a racemic mixture of the thioether lipid are good candidate drugs for the clinical trials. In fact, **3b** (Cytoros) is highly active in both human colorectal cancer xenografts (11) and PSN-1 human pancreatic cancer xenograft in nude mice (12), in addition to the broad spectrum of antitumor activity against *in vivo* animal solid tumor models (4,5,8,9). Furthermore, this compound inhibited liver metastases of M5076 sarcoma (8) and lung metastases of 3-Lewis lung carcinoma in mice (4,5). With regard to its action as prodrug of ara-C, Cytoros (**3b**) and its analogues are resistant to hydrolysis by cytidine deaminase (29), are taken up rapidly by tumor cells (30), give a greater intracellular retention of ara-CTP than that resulting from ara-C (8,12) and interact extensively with serum lipoproteins (31). In addition, superior antitumor activity of Cytoros as compared to *sn*-1 isomer of ara-CDP-dipalmitin, a diacylglycerol conjugate (6), could possibly be due, in part, to the release of two cytotoxic groups, ara-CTP and 1-*S*-thioether lysophospholipid after biotransformation (32,33). A preclinical toxicology study of Cytoros is now in progress.

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Synthesis of Oxygenated Fatty Acid Esters from Santalbic Acid Ester

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The reaction of methyl octadec-*trans*-11-en-9-ynoate (1) with mercuric sulfate in the presence or absence of sulfuric acid is described. Treatment of 1 with mercuric sulfate in absolute methanol yielded methyl 9(10)-oxooctadec-*trans*-11-enoates (Product A). This product, upon treatment with *m*-chloroperbenzoic acid, afforded methyl *trans*-11,12-epoxy-9-oxooctadecanoate (4) and methyl 10-oxooctadec-*trans*-11-enoate (2). Sodium borohydride reduction of A furnished the corresponding hydroxy esters. The treatment of 1 with mercuric sulfate in the presence of sulfuric acid gave as major product methyl 9(10)-oxo-11(12)-methoxyoctadecanoates and methyl 9(10)-oxooctadec-*trans*-11-enoates as a minor product. When methyl 11,12-epoxyoctadec-9-ynoate was reacted with acid in methanol, methyl 12-hydroxy-11-methoxyoctadec-9-ynoate was formed, which on treatment with zinc chloride in CCl_4 yielded methyl 9,12-epoxyoctadec-9,11-dienoate exclusively. The preparation of oxo fatty esters from the total methyl esters of *Santalum album* was also demonstrated. The structures of the products were established by chemical derivatization and spectral characterization. *Lipids 28,* 1027–1031 (1993).

Santalbic (octadec-*trans*-11-en-9-ynoic) acid, a conjugated enynoic fatty acid, is a major component of *Santalum album* (Linn.) seed oil. The seeds from *S. album* contain about 50–60% drying oil, which is 74% santalbic acid (1). The seeds can be collected in quantity from sandal wood plantations. Santalbic acid can be readily isolated in high yield by crystallization from mixed fatty acids of *S. album* seed oil. Although large amounts of santalbic acid are produced naturally, few studies (1–5) have been undertaken on the chemistry of this fatty acid.

The chemistry of conjugated enynes is of current interest for the synthesis of biologically active compounds such as pheromones, leukotrienes and prostaglandins (6–8). Lam and Lie Ken Jie (9,10) have synthesized methyl 5(6)-oxooctadec-*cis*-10-enoates and 5(6)-hydroxy-10(11)-methoxyoctadecanoates by oxymercuration and demercuration reactions of a nonconjugated enynoic fatty ester (methyl octadec-*cis*-10-3-en-5-ynoate). Earlier workers have reported the hydration of nonconjugated enynic compounds with sulfuric acid and mercuric sulfate (11).

To the best of our knowledge, no such work has been carried out on long-chain fatty acids containing conjugated enynic chromophores. To explore the utility of santalbic acid, we have carried out some studies on its oxidation reactions. Santalbic acid esters were modified to α- and β-enoates, 9(10)-oxo-11(12)-methoxy fatty esters, 11,12-epoxy-9-oxo fatty ester, 11,12-epoxy-9-ynoate, 12-hydroxy-11-methoxy-9-ynoate and a furanoid fatty acid ester.

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Abbreviations: GLC, gas-liquid chromatography; IR, infrared; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Thin-layer chromatography (TLC) was done on microscope glass plates coated with silica gel G (about 0.1 mm thick), and a mixture of petroleum ether (40–60°C)/diethyl ether in various proportions was used as developing solvent. Perchloric acid (40% in water) was used as spray reagent to visualize fractions on the plates. Column chromatography was performed on silica (60–120 mesh) using gradient elution with a mixture of petroleum ether/diethyl ether. Collected fractions were checked by TLC to ascertain their purity. Adsorbents for TLC and column chromatography were obtained from E. Merck Limited (Bombay, India). All reagents and solvents were of analytical grade; solvents were distilled and dried before use. Sulfuric acid (95–98%), sodium borohydride, sodium bicarbonate, perchloric acid and zinc chloride were purchased from E. Merck, and *m*-chloroperbenzoic acid was from Fluka (Buchs, Switzerland). Mercuric sulfate was obtained from E. Merck (Darmstadt, Germany).

S. album seeds were obtained from Sandal Research Centre (Bangalore, India). Infrared (IR) spectra were measured on a Shimadzu IR-408 Spectrometer (Tokyo, Japan). Mass spectroscopy (MS) was done on a JEOL JMS-D3000 instrument (Tokyo, Japan) at 70 eV. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian (Palo Alto, CA) A 60D spectrometer. NMR spectra were measured on $CDCl_3$ solutions and chemical shifts are reported in parts per million downfield from tetramethylsilane as internal standard.

Extraction of the oil from S. album *seeds, isolation of santalbic acid and preparation of its methyl ester. S. album* seeds were crushed in a mortar to separate kernels from the pericarp. The kernels and sodium sulfuate (20 g/50 g of kernels) were then finely crushed, and oil was extracted by repeated addition of petroleum ether (40–60°C) in a Soxhlet apparatus on a waterbath for 3 h. The petroleum ether extract obtained from the kernels was kept on anhydrous sodium sulfate overnight and was then filtered. The removal of petroleum ether under reduced pressure yielded 60% of a lightly yellowish oil. The oil (32.7 g) was hydrolyzed by refluxing with 120 mL of ethanolic potassium hydroxide (6.54 g/130.8 mL) solution for 1 h. During this saponification process, a gummy substance was separated from the reaction mixture. Ethanol was then evaporated *in vacuo* at 60°C. The potassium salts of the mixed fatty acids were dissolved in excess water and acidified with 1N HCl. The liberated free fatty acids were extracted repeatedly with diethyl ether. The combined extract was dried over anhydrous sodium sulfate, and the solvent was evaporated, yielding 30.0 g of mixed free fatty acids.

The mixed fatty acids (30.0 g) of *S. album* seed oil upon crystallization from hexane at −10°C yielded crude santalbic acid (22.4 g; m.p. 36–38°C), which upon recrystallization from hexane at −10°C, gave the pure product [×9.7 g; m.p. 39°C; m.p. reported (2) 38.5°C]. Methyl santalbate (1) was prepared by treating the santalbic acid (4 g) overnight with a 50-mL mixture of methanol/benzene/sulfuric

acid (21:2.5:1, by vol) at room temperature. Water (approximately 100 mL) was added to the reaction mixture to reach the cloud point. The methyl ester was extracted repeatedly with diethyl ether, and the ethereal layer was washed twice with exceses 5% (wt/vol) sodium bicarbonate solution, then twice with water. The ethereal layer was dried over anhydrous sodium sulfate and the diethyl ether was evaporated yielding methyl santalbate. The purity of methyl santalbate was verified by TLC and gas-liquid chromatography (GLC) (99% pure).

Reaction of 1 *with mercuric sulfate and absolute methanol.* Compound 1 (1.15 g) was added to a well-stirred solution of mercuric sulfate (0.227 g) in absolute methanol (30 mL) at room temperature. The reaction was complete within 30 min. The reaction mixture was diluted by adding excess water (about 100 mL) and then extracted with diethyl ether (3 × 20 mL). The ethereal layer was dried over anhydrous sodium sulfate, filtered and the solvent was evaporated to yield a lightly yellowish oily product which on silica gel column chromatography afforded Product A in 95% yield. ^{1}H NMR spectrum ($CDCl_3$) δ 6.46-7.0 ppm (*m*, C*H*=C*H*CO), 5.5-5.83 (*m*, C*H*=C*H*CH$_2$CO), 3.0 (*d*, CH=CHC*H*$_2$CO), 3.58 (*s*, COOC*H*$_3$) 2.1-2.45 (*m*, methylene protons α to ester carbonyl, free carbonyl and double bond). IR spectrum (film) 1740 (s, ester carbonyl), 1710 (s, C=O stretching), 1670 and 1630 (s, CH=CHCO), 950 (s, *trans*-unsaturation) cm^{-1}; mass spectrum (*m/z*, rel intensity) 310 (M^+, absent), 225 ($M^+ - R^1$, 52.3), 185 ($M^+ - R^1CH{=}CHCH_2$, 57.1), 125 ($M^+ - R^2CO$, 38.09), 199 ($M^+ - R^1CH{=}CH$, 21.4), 153 ($M^+ - R^2$, 26.1), 139 ($M^+ - R^2CH_2$, 52.3), 280 (9.52), 279 (45.2), 278 (4.76), 267 (2.3), 226 (0.7), 211 (9.5), 200 (4.7), 55 (base peak, 100).

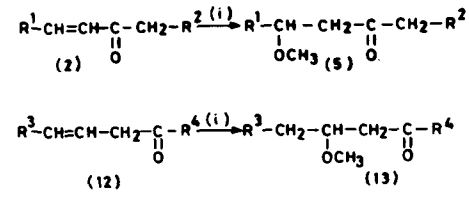
Reaction of Product A *with* m-*chloroperbenzoic acid.* Product A (0.2 g) was dissolved in chloroform (50 mL), and *m*-chloro-perbenzoic acid (0.71 g) was added in portions. The reaction was allowed to run for 24 h at 5°C. Chloroform was removed from the reaction mixture under reduced pressure, and the reaction products were extracted with diethyl ether (3 × 20 mL). Removal of diethyl ether and subsequent silica gel column chromatography of the reaction product yielded about 70% of methyl 10-oxo-octadec-*trans*-11-enoate (2) and 25% of methyl 9-oxo-*trans*-11,12-epoxyoctadecanoate (4). Product 2: R_f, 0.69; ^{1}H NMR spectrum ($CDCl_3$) δ 6.46-7.0 ppm (*m*, 2H, C*H*=C*H*CO), 3.6 (*s*, 3H, COOC*H*$_3$), 2.0-2.6 (*m*, 6H, methylene protons α to ester carbonyl, free carbonyl and double bond); IR spectrum (film) 1740 (s, ester carbonyl), 1670 and 1630 (s, CH=CHCO) cm^{-1}. Product 4: R_f, 0.53; ^{1}H NMR spectrum ($CDCl_3$) 2.9-3.0 (*m*, 2H, epoxide protons), 3.6 (*s*, 3H, COOC*H*$_3$), 2.2-2.6 (*m*, 6H, methylene protons α to ester carbonyl, free carbonyl and epoxy ring), 1.3 (*m*, chain methylene protons) and 0.9 (*t*, 3H, terminal methyl protons). IR spectrum (film) 1740 (s, ester carbonyl), 890 (s, 1,2-epoxide stretching), 1710 (s, C=O stretching).

Reaction of 1 *with mercuric sulfate and methanol containing sulfuric acid.* Methyl santalbate (1) (1.15 g) was added to a well-stirred solution of mercuric sulfate (0.227 g), sulfuric acid (1.55 g) and absolute methanol (30 mL). The reaction mixture was stirred for 90 min at room temperature. After completion of the reaction, water (100 mL) was added to the reaction mixture, and the mixture was extracted with diethyl ether (3 × 20 mL). The ethereal layer was successively washed with dilute $NaHCO_3$ (10%, 40 mL) and water (2 × 40 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated. Silica gel column chromatography of the reaction mixture yielded two products, D as the major (70%) and C as the minor product (25%). The major Product D gave the following spectral data: ^{1}H NMR spectrum ($CDCl_3$) δ 3.7 ppm (*s*, 3H, COOC*H*$_3$), 3.3 (*s*, 3H, HC-OC*H*$_3$), 2.16-2.6 (*m*, 6H, methylene protons α to ester carbonyl and free carbonyl), 3.6 (*m*, 1H, methine); IR spectrum (film) 1740 (s, ester carbonyl), 1710 (s, C=O stretching), 1160 (m, ether linkage) cm^{-1}; mass spectrum (*m/z*, rel intensity) 342 ($M^+ - R^1CH_2CHOCH_3$, 92.8), 143 ($M^+ - CH_2COR^2$, 15.47), 243 ($M^+ - R^1CH_2$, 32.14), 171 ($M^+ - R^1CH(OCH_3)CH_2CO$, 44.04), 257 ($M^+ - R^1$, 100), 295 (11.9), 296 (14.2), 280 (4.7), 279 (27.3), 258 (14.2), 227 (52.3), 228 (8.3), 200 (13.0), 183 (2.3), 139 (38.09), 129 (67.85), 55 (base peak, 100). The minor Product C was identical with Product A.

Sodium borohydride reduction of Product A. Sodium borohydride (0.025 g) was added in portions within 10 min at 0°C to a well-stirred solution of A (mixture of 2 and 3) in methanol (0.2 g in 25 mL). Monitoring of the reaction mixture by TLC indicated the completion of the reaction within 15 min. The reaction mixture was diluted immediately by adding excess water, acidified with 1N HCl and extracted with diethyl ether. Evaporation of diethyl ether gave the oily Product E (a mixture of 7 and 8), which was purified by silica gel column chromatography. ^{1}H NMR spectrum ($CDCl_3$) δ 5.33-5.5 ppm (*m*, olefinic protons), 3.9-4.38 (*m*, methine protons), 3.6 (*s*, 3H, COOC*H*$_3$), 1.93 (*s*, -O*H*, D_2O exchangeable); IR spectrum (film) 1740 (s, ester carbonyl), 3350 (broad, -OH), 965 (*m*, *trans*-unsaturation) cm^{-1}. Interconversion of Product E into Product A was achieved by chromic acid oxidation (12); Product A was identified as a mixture of 2 and 3 by spectral analysis.

Reaction of 2 *and* 12 *with methanol containing sulfuric acid.* Methyl 12-oxo-octadec-9-enoate (9) was prepared from methyl 12-hydroxy-octadec-9-enoate (12).

The compounds 2 and 12 (0.5 g each) were each dissolved separately in 30 mL of methanol containing sulfuric acid (1.55 g) and stirred for 90 min at room temperature. The reaction mixture was diluted by adding 100 mL of water, and the reaction products were extracted with diethyl ether. The products were purified by silica gel column chromatography using petroleum ether/diethyl ether (85:15, vol/vol) as eluent. Compound 2 furnished Product 5 (70%), and 12 afforded Product 13 (70%) (Scheme 1).



SCHEME 1

Product 5: ^{1}H NMR spectrum ($CDCl_3$) δ 3.7 ppm (*s*, 3H, $COOCH_3$), 3.3 (*s*, 3H, $CH-OCH_3$), 2.16-2.6 (*m*, 6H, methylene protons α to ester carbonyl and free carbonyl) 3.6 (*m*, 1H, methine); IR spectrum (film) 1740 (s, ester carbonyl), 1710 (s, C=O stretching), 1160 (*m*, ether linkage) cm^{-1}; mass spectrum (*m/z*, rel intensity) 257 ($M^+ - R^1$, 100), 129 ($M^+ - R^2CH_2COCH_2$, 99), 199 ($M^+ - R^1CH(OCH_3)CH_2$, 93), 171 ($M^+ - R_2CH_2$, 49), 185 ($M^+ - R^2$, 98), 327 (3.7), 311 (5.8), 295 (6.4), 279 (7.1), 258 (3.9), 244 (6.4), 243 (19.0), 225 (14.9), 212 (3.6), 200 (14.5), 186 (17.0), 172 (5.7), 168 (10.4), 154 (21.0), 153 (18.6), 149 (21.7), 143 (42.4), 139 (60.5), 130 (10.5), 127 (19.6), 125 (50.4), 121 (23.3), 55 (base peak, 100). Product 13: ^{1}H NMR spectrum ($CDCl_3$) δ 3.7 ppm (*s*, 3H, $COOCH_3$), 3.3 (*s*, 3H, $HC-OCH_3$), 2.16-2.6 (*m*, 6H, methylene protons α to ester carbonyl and free carbonyl), 3.6 (*m*, 1H, methine); IR spectrum (film) 1740 (s, ester carbonyl), 1710 (s, C=O stretching), 1160 (m, ether linkage) cm^{-1}; mass spectrum (*m/z*, rel intensity) 342 (M^+, absent), 257 ($M^+ - R^4$, 95), 113 ($M^+ - R^3CH_2CH(OCH_3)CH_2$, 80), 229 ($M^+ - R^4CO$, 84), 127 ($M^+ - R^3CH_2CH(OCH_3)$, 30.7), 183 ($R^3CH_2CH(OCH_3) - CH_3OH$, 10.1), 171 ($M^+ - R^3CH_2$, 11.9), 283 (5.1), 264 (12.6), 230 (13.4), 205 (2.2), 202 (4.8), 199 (6.6), 195 (2.2), 185 (4.6), 180 (4.4), 179 (8.9), 161 (11.2), 158 (5.9), 143 (14.4), 137 (11.3), 115 (33.1), 109 (32.3), 101 (28.0), 55 (base peak, 100).

Preparation of oxo fatty esters from total methyl esters of S. album *seed lipids.* Total methyl esters of *S. album* seed lipids (1.15 g) were added to a well-stirred solution of mercuric sulfate (0.167 g) in absolute methanol (30 mL). After half an hour, the reaction was stopped by adding excess water (about 100 mL), and the reaction products were extracted (3 × 20 mL) with diethyl ether. The combined ethereal layer was dried over anhydrous sodium sulfate. Solvent was evaporated, and the oxygenated fatty esters were separated from nonoxygenated fatty esters by silica gel column chromatography to afford 72% of Product A.

Reaction of methyl 11,12-epoxyoctadec-9-ynoate (9) with acidic methanol. Methyl 11,12-epoxy-octadec-9-ynoate (9) was synthesized by the procedure of Abbot and Gunstone (13). The compound 9 (1 g) was stirred vigorously with 15 mL of 10% aqueous sulfuric acid in methanol. After 5 min, TLC of the reaction mixture showed the complete conversion of epoxide into a more polar compound. The reaction mixture was diluted by adding excess water (100 mL), and the product was extracted with diethyl ether. The organic layer was washed twice with $NaHCO_3$ (10%, 40 mL) and then with water (2 × 40 mL) and dried over anhydrous sodium sulfate. Evaporation of the solvent yielded an oily product, which was purified over a silica gel column to remove some minor contaminants. The isolated compound (95%) was identified as methyl 12-hydroxy-11-methoxy-octadec-9-ynoate (10) on the basis of the following spectral data: ^{1}H NMR spectrum ($CDCl_3$) δ 3.88 ppm (*m*, 2H, methine), 3.66 (*s*, 3H, $COOCH_3$), 3.43 (*s*, 3H, OCH_3), 2.63 (*s*, 1H, $-OH$, D_2O exchangeable), 2.3 (*m*, 4H, methylene protons α to ester carbonyl and triple bond); IR spectrum (film) 1740 (s, ester carbonyl), 3350 (broad, -OH), 1160 (*m*, ether linkage) cm^{-1}.

Reaction of methyl 12-hydroxy-11-methoxy-octadec-9-ynoate (10) with zinc chloride. Compound 10 (1 g), carbon tetrachloride (30 mL) and zinc chloride (0.4 g) were refluxed for 2 h. TLC of the reaction mixture revealed the formation of only one product. Carbon tetrachloride was removed under reduced pressure, and the product was extracted with diethyl ether (3 × 20 mL). The combined ethereal layers were washed twice with water. Diethyl ether was evaporated, and the product was purified by column chromatography. Based on spectral data, the product (90%) was confirmed to be methyl 9,12-epoxyoctadec-9,11-dienoate (11). ^{1}H NMR spectrum ($CDCl_3$) δ 5.78 ppm (*s*, 2H, ethylenic), 3.6 (*s*, 3H, $COOCH_3$), 2.08-2.66 (*m*, 6H, methylene protons α to ester carbonyl and furan ring); IR spectrum (film) 1740 (s, ester carbonyl), 1555 (s, $\nu_{C=C}$, furan stretching), 1015 (furan ring breathing) cm^{-1}.

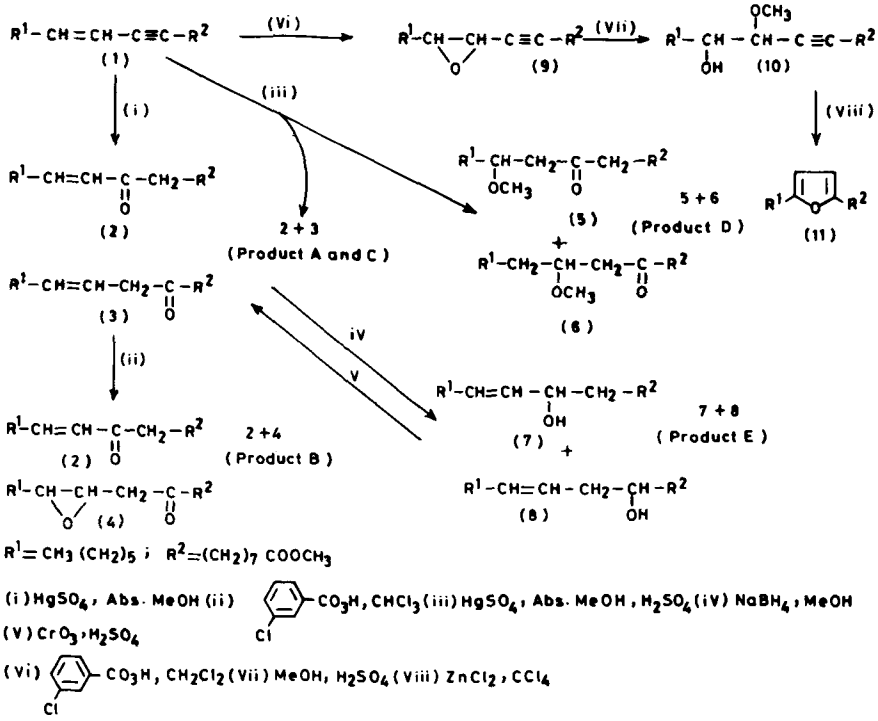
RESULTS AND DISCUSSION

Reaction of 1 with mercuric sulfate in absolute methanol (Scheme 2) gave Product A, which is a mixture of methyl 10-oxo-octadec-*trans*-11-enoate (2) and methyl 9-oxo-octadec-11-enoate (3). The same reaction in the presence of sulfuric acid (Scheme 2) resulted in the formation of 2 and 3 in minor amounts (Product C) and 5 and 6 (methyl 9(10)-oxo-11(12)-methoxy-octadecanoates) in major amounts (Product D). The hydration of 1 in the presence of sulfuric acid yielded, initially 2 and 3. Compound 2, an α,β-unsaturated oxo fatty ester, was readily converted to methyl 10-oxo-12-methoxy octadecanoate (5) by sulfuric acid catalyzed methoxylation. The β,γ-unsaturated oxo fatty ester (3) was first converted to α,β-unsaturated oxo fatty ester (methyl 9-oxooctadec-10-enoate), which on further acid-catalyzed methoxylation yielded methyl 9-oxo-11-methoxy-octadecanoate (6). These observations were supported by the acid-catalyzed methoxylation of pure α,β-unsaturated oxo fatty ester (2) and β,γ-unsaturated oxo fatty ester (12), which afforded β-methoxy ketones (5 and 13), respectively (Scheme 1).

Although compounds 2 and 3 of Product A were not separable by TLC, their identity was established by spectral characterization. The NMR spectrum of the mixture exhibited a complex multiplet at δ 6.46-7.0, which is characteristic of the α- and β-protons of α,β-unsaturated keto moiety. A broad multiplet at δ 5.5-5.83 for two protons (β and γ) and a doublet at δ 3.0 for two methylene protons of C-10 suggested the presence of a β,γ-unsaturated carbonyl system. The IR spectrum displayed characteristic bands at 1670 and 1630 cm^{-1}, which are due to the presence of an α,β-unsaturated carbonyl group, and at 1710 cm^{-1} indicating the presence of a β,γ-unsaturated carbonyl function. The mass spectral fragmentation pattern of Product A was in good agreement with the structures unveiled by IR and NMR spectra, which exhibited the structure identifying fragment ions at *m/z* 185 ($M^+ - R^1CH{=}CHCH_2$, 57.1) and *m/z* 139 ($M^+ - R^2CH_2$, 52.3), suggesting that Product A was a mixture of 2 and 3.

Compounds 2 and 3 of reaction Product A were separated when the mixture was treated with *m*-chloroperbenzoic acid at 5°C. Compound 3 readily formed an epoxide (4) while 2 remained unchanged, which greatly facilitated their separation by chromatography.

In the presence of sulfuric acid, the reaction of 1 with methanolic mercuric sulfate yielded C as the minor product and D as the major product. Product D was identified by IR, ^{1}H NMR and MS as a mixture of methyl 10-oxo-12-methoxy-octadecanoate (5) and methyl 9-oxo-11-methoxy-octadecanoate (6).



SCHEME 2

Sodium borohydride reduction of Product A (Scheme 2) at 0°C afforded methyl (9)10-hydroxy-octadec-11-enoates (7 and 8), which could find importance in the preparation of low-temperature plasticizers. It has been reported (14) that superior low-temperature plasticizing properties can be obtained with fatty acids having a hydroxyl group at C-9 or C-10 rather than C-12 position as in ricinoleic acid.

The mercuric sulfate-catalyzed reaction of santalbic acid is a useful method for the preparation of oxo fatty esters (2 and 3) in high yield. These oxo fatty esters can also be obtained directly from the mixed fatty acid methyl esters of *S. album* seed oil in 70% yield.

Lie Ken Jie and Lam (15) described the synthesis of isomeric furanoid C_{18} acids from furan and furfural or appropriate octadecadiynoates. Alternatively, furanoid acids have been prepared from appropriate C_{18} compounds by reactions which include Pd(II)-catalyzed cyclodehydration of unsaturated oxygenated acids and rearrangement of unsaturated oxygenated acids (16). The present study describes a new method for synthesizing methyl 9,12-epoxy-octadec-9,11-dienoate (**11**), a substance found naturally in fish and other lipids (17). The furanoid ester (**11**) has been synthesized from the α,β-acetylenic epoxide (**9**) in two steps (Scheme 2). The acid-catalyzed ring opening reaction of **9** yielded, exclusively, 11-methoxy-12-hydroxy-ynoate (**10**), which on subsequent cyclization under the influence of zinc chloride afforded methyl 9,12-epoxy-octadec-9,11-dienoate (**11**). The sharp NMR signal exhibited by this compound at δ 5.78 (two ethylenic protons) and characteristic IR bands at 1555 and 1015 cm^{-1} suggested it to be a furanoid fatty ester. This furanoid fatty ester was earlier prepared from α,β-epoxy acetylenic ester by mercuric sulfate catalyzed cyclization (13).

The ring opening reaction of epoxide (**9**) with acidic methanol may yield an isomeric mixture of 11(12)-hydroxy-12(11)-methoxy-ynoates. We observed the formation of the 12-hydroxy-11-methoxy-ynoate (**10**) but not of 11-hydroxy-12-methoxy-ynoate isomer. This was confirmed by the ready formation of furanoid fatty ester (**11**) on treatment of compound **10** with zinc chloride. The reason for the exclusive formation of isomer **10** could also be explained on the basis of the stabilities of the intermediate carbocations. Isomer **10** was formed by the intermediacy of propargylic cation (-CH(OH)$\overset{+}{C}$HC≡C-), which is much more stable (18) as compared to the other expected cation (-$\overset{+}{C}$HCH(OH)C≡C-).

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METHOD

Application of Paired-Ion High-Performance Liquid Chromatography to the Separation of Molecular Species of Phosphatidylinositol

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A paired-ion high-performance liquid chromatographic (HPLC) procedure was developed for the separation of individual molecular species of phosphatidylinositol (PI) without prior derivatization. The separation is accomplished on a reverse-phase column using acetonitrile, methanol and 10 mM tetrabutylammonium phosphate as mobile phase. Separation of the major molecular species of soybean PI by HPLC is achieved within 60 min. The method is applicable to analytical and metabolic studies on PI and has the advantage that the entire PI structure is preserved, as no degradation or derivatization procedures are involved.
Lipids 28, 1033–1035 (1993).

Phospholipids consist of populations of well-defined molecular species that contain various fatty acyl chains at both the 1- and 2-position of the glycerol backbone (1,2). The molecular species composition of phosphatidylinositol (PI) in mammalian tissues is quite different from that of other phospholipids, as there is a great abundance of the 1-stearoyl-2-arachidonoyl (18:0/20:4) species in PI (1). The rapid turnover of PI and of polyphosphoinositides in response to extracellular stimuli is believed to be part of the signal transduction system in various mammalian tissues (3,4).

The metabolic heterogeneity of PI molecular species has been studied in liver and brain using radiolabeled precursors, such as [^{3}H]glycerol and [^{32}P]phosphate (5,6), and preferential degradation of the arachidonoyl species of phospholipids, including those of PI, in response to extracellular stimuli has been reported in collagen-stimulated platelets (7) and in concanavalin A-stimulated lymphocytes (8). These studies have reemphasized the importance of monitoring the metabolism of PI at the molecular species level. However, little information is available about PI molecular species in mammalian tissues as the separation of phospholipid species with uniform structure is difficult to achieve.

Reverse-phase high-performance liquid chromatography (HPLC) is a useful method for the separation of phospholipid molecular species. We have previously developed an HPLC method for the separation of diradylglycerol derivatives from phospholipids, after derivatization to 1,2-diacyl-3-acetylglycerols (9), and several methods are available for the separation of molecular species after modification at the 3-position of the glycerol backbone (10–12). However, these methods are typically less suited for studies in which metabolic changes in phospholipid polar head groups occur because the polar heads are removed prior to analysis. Patton *et al.* (13) developed an HPLC method for the separation of phospholipid molecular species without modification of polar head groups; however, this method is time-consuming. It is also generally difficult to achieve a clear separation of intact phospholipids by reverse-phase HPLC because of the presence of the free polar head groups. Thus a reduction in the polarity of the polar head group is desirable to achieve a better separation.

Ion-pair reverse-phase HPLC has been successfully employed for the separation of both cationic and anionic compounds, such as barbiturates, sulfonamides and 5-fluorouracil (14–16). PI is an anionic hydrophobic compound, and cationic ion-pair reagents, such as tetrabutylammonium (TBA) or hexadecyltrimethylammonium ions, can counter the charge of the PI phosphate groups through the formation of ion-pairs with PI. Counter ions thus can enhance nonpolar interactions of the PI moiety in the reverse-phase system, leading to increased selectivity and improved separation.

The present report describes the first application of an ion-pair reagent to the separation of the molecular species of PI by reverse-phase HPLC.

EXPERIMENTAL PROCEDURES

Materials. TBA phosphate was obtained from Kodak (Rochester, NY). Soybean PI was purchased from Sigma (St. Louis, MO) and purified by thin-layer chromatography using chloroform/methanol/acetone/acetic acid/water (5:1:2:1:0.5, by vol) as developing solvent. HPLC-grade organic solvents were purchased from Wako Pure Chemical Co. (Osaka, Japan).

Apparatus. The chromatographic system included a model 6-A pump (Shimadzu Co., Kyoto, Japan) and a model SPD-6A ultraviolet (UV) absorbance detector (Shimadzu Co.). PI was dissolved in chloroform/methanol (1:1, vol/vol), and a glass syringe with a 0.45-μm membrane filter (type HV; Nihon Millipore Kogyo K.K., Tokyo, Japan) was used to clean the samples prior to injection. PI was loaded onto the reverse-phase column (LiChrosorb RP-18; 25 × 0.4 cm i.d.; particle size, 5 μm; Merck, Darmstadt, Germany) and eluted with an isocratic mobile phase (acetonitrile/methanol/10 mM TBA phosphate, 51:35:14, by vol) at a flow rate of 0.5 mL/min. The column temperature was kept at 35°C using a heating box (model CTO-6A; Shimadzu Co.). The eluate was monitored by measuring the UV absorbance at 205 nm; absorbance was recorded with an integrator (model C-R 3A; Shimadzu Co.).

Fatty acid analysis. Fractions containing PI molecular species after elution from the reverse-phase column were transmethylated by treatment with 0.5 M sodium methoxide; fatty acyl moieties were identified as their methyl-esters by gas chromatography (GC) on a GC-14A instrument (Shimadzu Co.) using a capillary column (30 m × 0.53 mm i.d.; Supercowax X1; Supelco Japan, Tokyo,

Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; PI, phosphatidylinositol; TBA, tetrabutylammonium, UV, ultraviolet.

Japan). Molecular species were quantified relative to heptadecanoic acid methyl ester, which was used as an internal standard.

RESULTS AND DISCUSSION

Soybean PI are particularly suited to demonstrate the analysis of molecular species because, unlike mammalian PI, they contain a variety of fatty acids. Soybean PI was separated into individual molecular species by paired-ion HPLC within 60 min. The solvent system, based on acetonitrile, methanol and TBA phosphate, proved useful for the separation of molecular species by reverse-phase without any prior modification. Although isopropyl alcohol is commonly added to solvent systems in reverse-phase HPLC, it did not improve the separation in our situation. TBA phosphate markedly enhanced the interaction of PI with the particle surface of reverse-phase column and increased observed retention times. Soybean PI was resolved into several fractions under the present isocratic conditions (Fig. 1). Analysis by gas–liquid chromatography of individual peaks revealed that seven different constituents of soybean PI were separated from each other (Table 1). The composition of soybean PI was quite different from that of PI from mammalian tissues in that the 18:0/20:4 species was only a minor component of soybean PI. The predominant molecular species of soybean PI were 16:0/18:2 (55.4%) and 18:0/18:2 (15.2%). The retention time of the molecular species increased with increasing chainlength and decreased with increasing degree of unsaturation of the fatty acids. The 18:0/18:1 species was expected to occur as a constituent of soybean PI, but was not found under the present conditions. This could be due to the low levels present and the long elution time of this species.

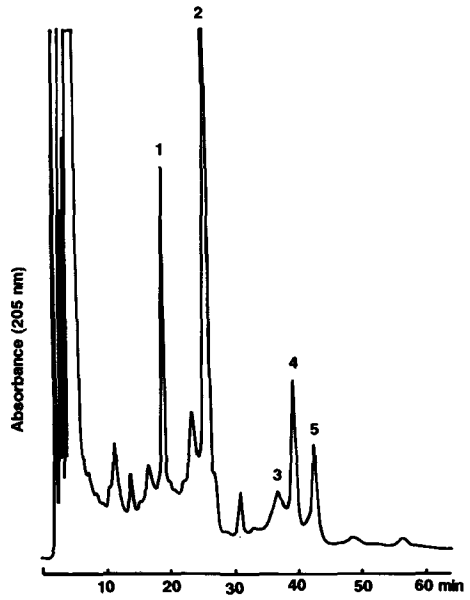

FIG. 1. Separation of the molecular species of soybean phosphatidylinositol (PI) by paired-ion high-performance liquid chromatography. Soybean PI, dissolved in 20 μL of chloroform/methanol (1:1 vol/vol), was chromatographed on a LiChrosorb RP-18 column (Merck, Darmstadt, Germany) at a flow rate of 0.5 mL/min. The mobile phase was acetonitrile/methanol/10 mM tetrabutylammonium phosphate (51:35:14, by vol). Eluates were monitored at 205 nm. The peak numbers correspond to those listed in Table 1.

TABLE 1

Relative Levels of the Individual Molecular Species of Soybean Phosphatidylinositol

Peak number[a]	Molecular species	Composition[b] (%)
1	16:0/18:3	7.1 ± 0.6
	18:2/18:2	7.0 ± 0.9
2	16:0/18:2	55.4 ± 3.6
3	16:0/18:1	6.0 ± 0.7
4	18:0/20:4	7.5 ± 0.4
	18:1/18:1	1.7 ± 0.1
5	18:0/18:2	15.2 ± 1.2

[a]Peak numbers correspond to the peaks in Figure 1.
[b]Values are expressed as the mean percentage ± SD (n = 3).

The fatty acid composition of PI, calculated from the molecular species composition as shown in Table 1, was similar to that of the original unfractionated sample, although the relative proportion of 18:1 was slightly lower after reverse-phase HPLC (Table 2). The results indicate that no significant losses of individual PI molecular species occurred during passage through the reverse-phase column.

The use of a TBA-containing eluent allowed the separation of PI molecular species in a manner that was not dependent, to any great extent, on the polarity of the head group. The method for the separation of the individual PI molecular species, as described here, will be valuable in studies on the metabolism of individual PI species. For example, the rates of turnover of the polar head group, of the acyl moieties and of the glycerol backbone of individual PI molecular species can be compared simultaneously by triple labelling, using [^{32}P]phosphate, [^{14}C]-glycerol and [^{3}H]fatty acid. In addition, the present method should also prove useful for the separation of the molecular species of polyphosphoinositides.

TABLE 2

Comparison of the Fatty Acid Composition of Phosphatidylinositol (PI) Before and After Reverse-Phase High-Performance Liquid Chromatography (HPLC)

Fatty acid	Original[a] (%[c])	HPLC[b] (%[c])
16:0	29.1 ± 1.3	32.1 ± 2.2
18:0	11.1 ± 0.8	10.7 ± 1.2
18:1	7.0 ± 0.4	4.4 ± 0.6
18:2	44.8 ± 2.1	44.9 ± 3.1
18:3	5.6 ± 0.3	4.4 ± 0.8
20:4	2.6 ± 0.2	3.5 ± 0.9

[a]Fatty acid composition of soybean PI.
[b]Proportions of fatty acids were calculated from the composition of the molecular species of PI presented (Table 1).
[c]Values are expressed as the mean percentages ± SD (n = 5).

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Separation and Identification of Isomeric Conjugated Fatty Acids by High-Performance Liquid Chromatography with Photodiode Array Detection

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A simple, high-performance liquid chromatographic method is described for the separation of tetraenoic, trienoic and dienoic conjugated fatty acids on a Zorbax ODS reversed-phase column using acetonitrile/tetrahydrofuran (95:5, vol/vol) at a flow rate of 1.2 mL/min as mobile phase. Also described is the separation of the isomeric conjugated fatty acids with acetonitrile/water/tetrahydrofuran (90:90:1, by vol) as mobile phase. The simultaneous detection and identification of the separated geometrical isomers in the eluant was accomplished using photodiode array detection. *Lipids 28*, **1037–1040 (1993).**

Conjugated fatty acids are an important class of compounds present in seed oils (1), and their separation, identification and quantitation is essential. Existing analytical methods are often tedious and time-consuming and usually involve a combination of procedures. High-performance liquid chromatography (HPLC) is commonly used to resolve fatty acids. However, only nonconjugated fatty acids have previously been analyzed by HPLC (for a review, see Ref. 2). The separation of positional and geometrical isomers can be accomplished by HPLC using the silver ion mode (3). The characterization of hydroxy/eicosatetraenoic acids and their conjugated dienes has been reported using ultraviolet (UV) spectroscopic detection (4). Tan (5) reported on a method for the analysis of carotenoids using nonaqueous reversed-phase HPLC and photodiode array (PDA) detection. The PDA detector has an advantage over other modes of HPLC detection, as it can selectively monitor UV chromophores of conjugated systems in mixtures. As each geometrical isomer has its characteristic λ_{max} value, PDA detection provides a convenient and reliable method for the identification of such isomers.

The conjugated fatty acids have distinct λ_{max} values in different regions of the spectrum, which are characteristic for their UV chromophores and facilitate identification. The conjugated tetraenoic acids (6–8) absorb in the range of 278–320 nm, whereas trienoic (9,10) and dienoic (11) fatty acids absorb around 260–285 nm and 225–245 nm, respectively. The purpose of the present study was to separate and identify isomeric conjugated tetraenoic, trienoic and dienoic fatty acids and to characterize the individual fatty acid isomers based on their geometrical configuration using HPLC and PDA detection.

MATERIALS AND METHODS

Materials. Garden balsum (*Impatiens balsamina*), bitter gourd (*Momordica charantia*) and snake gourd (*Trichosanthes anguina*) were obtained from the Seed Corporation (Hyderabad, India). Dehydrated castor oil (DCO) fatty acids were obtained from the Division of Oils and Fats (IICT, Hyderabad, India). All solvents were of HPLC grade (Spectrochem, Hyderabad, India).

Extraction procedure. The kernels of *I. balsamina*, *M. charantia* and *T. anguina* seeds were each ground with anhydrous Na_2SO_4 and were extracted three times with *n*-hexane (40–60°C) at room temperature. The combined extracts of each lipid sample were passed through anhydrous Na_2SO_4 and concentrated in a rotary evaporator at 40°C. The extracted oils were stored under nitrogen at −15°C.

Esterification. The fatty acid methyl esters from the oils were prepared by transesterification. The oil sample (100 mg) was dissolved in hexane (3 mL) in a round-bottom flask. Sodium methoxide in anhydrous methanol (5 mL) was added, the flask was flushed with nitrogen and the solution was shaken on a Griffin flask shaker for 30 min. Water (10 mL) was added, and the content was transferred into a separatory funnel and extracted with hexane (10 mL). The hexane layer was filtered through anhydrous Na_2SO_4. The methyl esters of DCO fatty acids were prepared by reaction with diazomethane (12). The methyl esters were purified by thin-layer chromatography (TLC) on Silica Gel G plates, using hexane/diethyl ether (90:10, vol/vol) as developing solvent, and stored under nitrogen at −15°C.

Preparation of standard samples. Methyl parinarate, methyl α-eleostearate and methyl punicate were isolated from the total methyl esters of *I. balsamina*, *M. charantia* and *T. anguina*, respectively. The total methyl esters of these oils were spotted onto Silica Gel G plates, and the plates were developed twice with hexane/diethyl ether (94:6, vol/vol) (13). The conjugated fatty esters were separated well from the nonconjugated ones, and the bands could be seen under UV light after spraying the plates with 2′,7′-dichlorofluorescein. The separated band of conjugated fatty acid esters was scraped off and extracted with diethyl ether by passing through a small column. The excess solvent was removed under vacuum, and the samples were stored under nitrogen at −15°C.

Isomerization of total methyl esters. An iodine solution (0.2 mL) was prepared by dissolving iodine in pure *n*-hexane (0.65 g/100 mL hexane) and was then added to the methyl esters (50 mg) dissolved in 3 mL of *n*-hexane. The reaction mixture was shaken well and left overnight at ambient temperature (14). The isomerized product was extracted with *n*-hexane and purified by passing through a small column of silica gel. β-Eleostearic acid was isolated from the isomerized methyl esters of *M. charantia* by preparative TLC using hexane/diethyl ether (94:6, vol/vol). β-Parinaric acid could not be isolated in pure form from the isomerized methyl esters of *I. balsamina*. Therefore, the isomerized methyl esters of *I. balsamina* were used as such for HPLC analysis. However, this did not affect the analysis, as the PDA detector facilitated the selective identification of the conjugated components, either from pure fatty acid esters or from total methyl esters at the

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Abbreviations: DCO, dehydrated castor oil; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; PDA, photodiode array; TLC, thin-layer chromatography; UV, ultraviolet.

wavelength corresponding to the conjugated tetraenoic fatty acids (278–320 nm).

UV spectroscopy. The λ_{max} values of pure fatty acid methyl esters were measured on 4.5 μg/mL solutions using a Shimadzu (Kyoto, Japan) UV-240 model recording double-beam spectrophotometer fitted with a deuterium lamp. The solvent used was cyclohexane. The λ_{max} values measured are given in Table 1.

HPLC equipment and conditions. HPLC separations were carried out using a Shimadzu LC-6A instrument with a single piston pump, a CDQR (Constant Displacement with Quick Return) system (Shimadzu, Tokyo, Japan) and a 7125 injector valve. The instrument was linked to MIA photodiode array UV-visible spectrophotometric detector (PDA detector) (Shimadzu) and a WX 4421 XYT C-R_3A data processor chromatopac recorder (Shimadzu). For all chromatographic separations, a 5-μm pore size 4.6 mm i.d. × 25 cm Zorbax ODS reversed-phase column from DuPont Instruments (Barley Mill Plaza, Wilmington, DE) connected to a Shimadzu 10-μm ODS guard column was used.

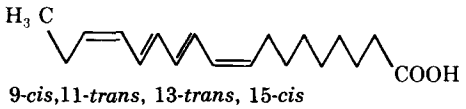
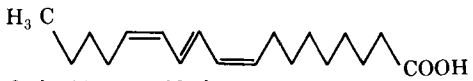
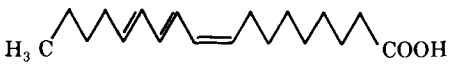
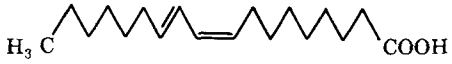
Sample preparation. To illustrate the separation and identification of individual conjugated tetraenoic and trienoic fatty acids, the methyl esters of *I. balsamina*, *M. charantia* and *T. anguina* oils were used. The isomerized methyl esters of *I. balsamina* oil were used to show the separation and identification of the geometrical isomers of conjugated tetraenoic acids. The methyl esters of DCO fatty acids were used for the separation and identification of the geometrical isomers of conjugated dienoic acids.

In all of the above cases, 5 mg of the sample was dissolved in 10 mL of the mobile phase (acetonitrile/tetrahydrofuran, 95:5, vol/vol) and used for analysis. However, for the separation and identification of the geometrical isomers of the conjugated trienoic acids, 2 mg each of the methyl esters obtained from *M. charantia* and *T. anguina* and the isomerized methyl esters of *M. charantia* oils were dissolved in 10 mL of the same mobile phase as above and used for the analysis.

Pure samples. One mg of each standard sample, *i.e.*, methyl parinarate, methyl α-eleostearate, methyl punicate

TABLE 1

Absorption Maxima of Conjugated Fatty Acids Measured by Photodiode Array Detection and by Conventional Ultraviolet Techniques

Conjugated fatty acid	UV[a] (nm)	Photodiode array detector[b] (nm)
Tetraenoic acids		
	280 292 303 320	281 293 304.5 321
9-*cis*,11-*trans*, 13-*trans*, 15-*cis* octadecatetraenoic acid		
	274 286 299 313	276 290 302.2 316
9-*trans*, 11-*trans*, 13-*trans*, 15-*trans* octadecatetraenoic acid		
Trienoic acids		
	265 275 287	267 276.5 287.5
9-*cis*, 11-*trans*, 13-*cis* octadecatrienoic acid		
	262 273 284	263 274.5 284.5
9-*cis*, 11-*trans*, 13-*trans* octadecatrienoic acid		
	259 268 279	261 270 281
9-*trans*, 11-*trans*, 13-*trans* octadecatrienoic acid		
Dienoic acids		
	232	236
9-*cis*, 11-*trans* octadecadienoic acid		
	230	234
9-*trans*, 11-*trans* octadecadienoic acid		

[a]Spectra were measured in cyclohexane.
[b]Spectra were measured in mobile phase.

and methyl β-eleostearate, was dissolved in 10 mL of the mobile phase and used for analysis.

Twenty μL of these solutions were injected using a 20-μL loop in the injector port. The conjugated fatty acids were eluted using a binary mobile phase consisting of acetonitrile/tetrahydrofuran (95:5, vol/vol) at a flow rate of 1.2 mL/min. The geometrical isomers were separated using the same column, but a more polar tertiary mobile phase consisting of acetonitrile/water/tetrahydrofuran (90:9:1, by vol), at a flow rate of 1.2 mL/min.

RESULTS AND DISCUSSION

Table 1 gives the λ_{max} values of the tetraenoic, trienoic and dienoic conjugated fatty acids and of their geometrical isomers as measured by PDA detection and conventional UV spectroscopy. The HPLC column clearly separated the conjugated tetraenoic and trienoic fatty acid analogues, but only partially resolved the dienoic fatty acid analogues. A small bathochromic shift was observed, which was thought to be a solvent effect.

Tetraenes. Figure 1 shows the three-dimensional spectrochromatogram of the conjugated tetraenoate, methyl parinarate, present in the methyl esters of *I. balsamina* oil (7,15). The mobile phase was acetonitrile/tetrahydrofuran (95:5, vol/vol), and the flow rate was 1.2 mL/min. Methyl esters of *I. balsamina* oil were injected onto the HPLC column at ambient temperature, and the eluant was monitored with the PDA detector at the fixed wavelength of 320 nm. The eluant was scanned over the 200–400 nm range. The resulting spectrochromatogram is shown in Figure 1. The λ_{max} at 304.5 nm (Fig. 1, inset) compared well with that of the conventional UV spectrum (λ_{max} 303 nm) and with the spectrochromatogram (304.5 nm) of pure methyl parinarate (methyl 9-*cis*,11-*trans*,13-*trans*,15-*cis*-octadecatetraenoate).

Figure 2 shows the spectrochromatogram of the isomers of methyl parinarate found in the eluant of the isomerized methyl esters derived from *I. balsamina* oil. The mobile

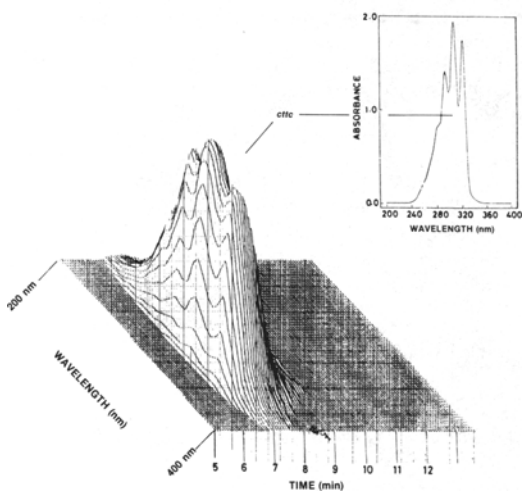


FIG. 1. Three-dimensional spectrochromatogram of tetraenoic fatty acid as seen by photodiode array detection. The inset shows the broad absorption band. A Zorbax ODS column and acetonitrile/tetrahydrofuran (95:5, vol/vol) as the mobile phase were used. The flow rate was 1.2 mL/min.

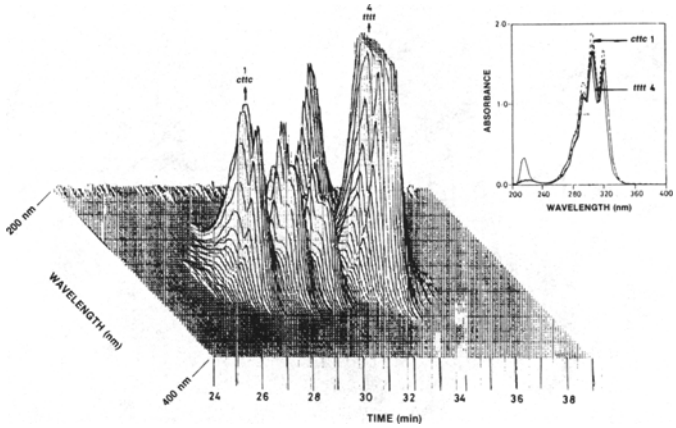


FIG. 2. Three-dimensional spectrochromatogram of the isomers of parinaric acid as seen by photodiode array detection. The inset shows the absorption spectra of the geometrical isomers. The mobile phase was acetonitrile/water/tetrahydrofuran (90:9:1, by vol) using the same column and flow rate as in Figure 1.

phase used here was acetonitrile/water/tetrahydrofuran (90:9:1, by vol), and the flow rate was 1.2 mL/min. Twenty μL of the sample was injected onto the HPLC column, and the eluant was monitored by PDA detection at an absorbance of 320 nm. Four isomers of parinaric acid can be seen in the broad absorption band in the range of 278–320 nm (Fig. 2, inset). The peak at λ_{max} 304.5 nm (peak 1) was identified as methyl parinarate (Fig. 1, inset). A similar spectrochromatogram with a λ_{max} 2.3 nm lower (302.2 nm) was assigned to the all-*trans* analogue, namely methyl β-parinarate, *i.e.*, methyl 9-*trans*,11-*trans*,13-*trans*,15-*trans*-octadecatetraenoate (peak 4). This assignment was confirmed by the relative retention times of methyl parinarate and β-parinarate in a separate analysis, where the isomerized methyl esters of *I. balsamina* oil were monitored using a UV detector and the column and conditions mentioned in Figure 2. The other two peaks seen in the center may be due to the other geometrical isomers of parinarate, as implied from their absorption profile (Fig. 2, inset), but their exact geometrical configuration could not be established.

Trienoates. The methyl esters of *M. charantia* and *T. anguina* were also subjected to HPLC separation at 270 nm using conditions similar to those given in Figure 1. The conjugated trienoate from *M. charantia* (13,16) was identified as methyl α-eleostearate. The UV spectrum (273 nm) and the spectrochromatogram (274.5 nm) of pure methyl α-eleostearate were used to further confirm methyl 9-*cis*,11-*trans*,13-*trans*-octadecatrienoate. Methyl punicate, namely methyl 9-*cis*,11-*trans*,13-*cis*-octadecatrienoate, from the methyl esters of *T. anguina* (13) oil had spectrum similar to that of methyl α-eleostearate with a λ_{max} 2 nm higher (at 276.5 nm). The results compared well with those obtained for pure methyl punicate by conventional UV and PDA detection. Figure 3 shows the spectrochromatogram of the three isomers, methyl punicate (peak 1), α-eleostearate (peak 2) and β-eleostearate (peak 3). The conditions were the same as those used in the separation illustrated in Figure 2. The three peaks corresponding to the *cis*,*trans*,*cis*(*ctc*),*cis*,*trans*,*trans*(*ctt*) and *trans*,*trans*,*trans*(*ttt*) analogues could be easily distinguished from

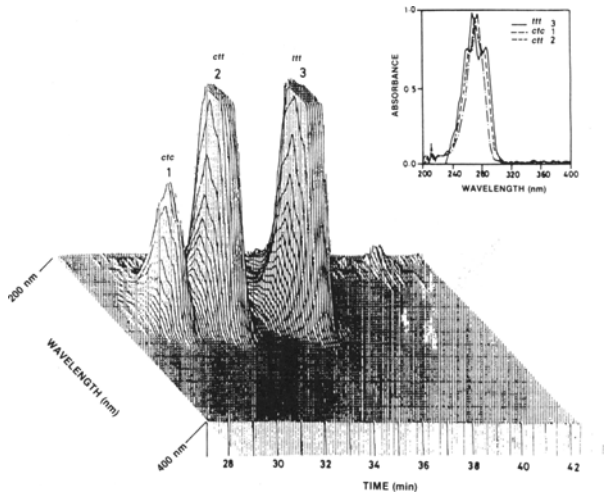


FIG. 3. Three-dimensional spectrochromatogram of the isomers of punicic acid as seen by photodiode array detection. The inset shows the absorption spectra of the isomers. Conditions are the same as in Figure 2.

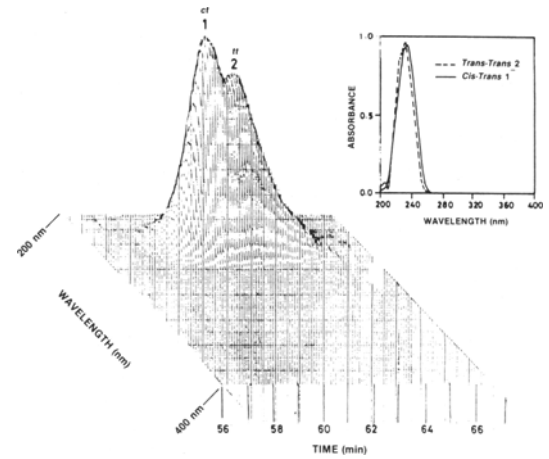


FIG. 4. Three-dimensional spectrochromatogram of the conjugated dienoic fatty acids as seen by photodiode array detection. The inset shows the absorption spectra. Conditions are the same as in Fig. 2.

each other. There were subtle, but noticeable differences in the chromophores of the individual isomers; and λ_{max} at 276.5, 274.5 and 270 nm confirmed the presence of all three geometrical isomers, methyl punicate, α-eleostearate and β-eleostearate, respectively, as shown in Figure 3. The all-*trans* analogue, β-eleostearate, had a similar spectrum with a λ_{max} 4.5 nm lower than that of its *ctt* analogue, α-eleostearate. The λ_{max} of methyl punicate was 2 nm higher than that of its *ctt* analogue, α-eleostearate (λ_{max} at 274.5 nm), and 6.5 nm higher than that of its all-*trans* analogue, β-eleostearate (λ_{max} 270 nm).

Dienes. Figure 4 presents the spectrochromatogram of the conjugated dienoates present in the methyl esters derived from DCO. The resolution was not quite satisfactory in this instance, as the retention times of the conjugated diene isomers were very close as seen from the spectrochromatogram obtained by PDA detection. However, under the experimental conditions employed for tetraenoic and trienoic acid isomers, only a partial separation of methyl 9-*cis*,11-*trans*-octadecanoate and 9-*trans*, 11-*trans*-octadecanoate analogues was possible. *cis*,*trans*-Dienoate had a λ_{max} 2 nm higher (λ_{max} 234 nm) than its *trans*,*trans* analogue (λ_{max} 232 nm. The HPLC method described, employing a PDA detector, is simple, rapid and sensitive. It permits the separation and simultaneous characterization of conjugated fatty acid isomers present in mixtures in a single analytical run.

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COMMUNICATION

Reevaluation of the Neutral Lipids of *Tilletia controversa* and *Tilletia tritici*

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Differential scanning calorimetry of whole teliospores and lipid extracts of *Tilletia controversa* Kühn and *T. tritica* Tul. indicated that the lipid composition of teliospores was different than earlier reported. An exothermic peak at −40 to −45°C and an endotherm at −25 to −15°C indicated that the majority of lipids were triacylglycerols (TAG). Hot isopropanol was used to inactivate lipases during lipid extraction. Thin-layer chromatographic analysis of extracted lipids showed that free fatty acids (FFA) were not present in great quantities unless water was present during lipid extraction. As measured by gas chromatography, FFA accounted for 1–5% of the lipid content in teliospores of *Tilletia* spp. The TAG content of teliospores was 60–80% of total lipids.
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Tilletia controversa Kühn (TCK) and *T. tritici* Tul. (TCT) are the fungi that cause dwarf and common bunts of wheat, respectively. A major difference between TCK and TCT is the germination of their teliospores. Teliospores of TCK take 4–6 wk to germinate while those of TCT require only 4–7 d (1). Additionally, TCK will only germinate at temperatures less than 7°C while TCT has an optimum germination temperature of 15°C. The cause of the dormancy of TCK has been elusive (1–3), but one theory has evolved around the high (30–35%) lipid content of teliospores (4,5). Gardner *et al.* (4) and others (5) reported that the predominant lipid class of these organisms was free fatty acids (FFA). It was postulated that FFA, in particular linoleic acid, and oxidation products from FFA, were the cause of dormancy of TCK and TCT (6).

Because the dwarf bunt mold is under quarantine regulations in several countries (7), this organism is important to wheat-exporting and -importing countries. Based on morphological characteristics, teliospores of TCK are difficult to differentiate from those of TCT (8). Our initial goal was to differentiate TCK from TCT by the use of differential scanning calorimetry (DSC) and supposed differences in lipid composition of teliospores. Preliminary DSC data indicated that the lipid profiles of these organisms were different than those reported. We report here on a reevaluation of the neutral lipids of TCK and TCT.

MATERIALS AND METHODS

Source of teliospores. Wheat spikelets infected by TCK or TCT were obtained from Blair Goates at the USDA, ARS, National Small Grains Germplasm Research Facility (Aberdeen, ID). Sori were separated from spikelets and crushed through a 90-nm sieve after outer plant material was removed. Lipids were extracted from a mixture of TCK races and two races of TCT (T-19 and T-4).

Lipid extraction. Throughout lipid extraction, great care was taken not to activate endogenous lipase. Dormant, dry teliospores were ground under a nitrogen atmosphere with glass beads agitated by a vortex mixer. Boiling isopropanol (9) was added before grinding to inactivate lipases. During homogenization, the mixture was periodically reheated to 85°C. Generally, it took 15–30 min of vortex mixing to achieve over 95% teliospore breakage using 50 to 100 mg of teliospores. After the isopropanol was evaporated from the homogenate, lipids were extracted with chloroform/methanol (1:2, vol/vol) by a modification of the method of Bligh and Dyer, as found in Kates (10). In all extractions, the aqueous phase was acidified to ensure complete extraction of FFA. Lipids were stored under nitrogen in either hexane or chloroform at −20°C. Three separate extractions were analyzed in duplicate.

DSC. A Thermal Analysis 2910 (TA Instruments, Groton, CT) equipped with a Liquid Nitrogen Cooling Accessory was used to determine crystallization and melting temperatures *in vivo* and of extracted lipids. Data were collected using a TA 2100 computer work station. Nitrogen gas was used to flush the cell during scans. Samples of teliospores or isolated lipids were held at 40°C for 20 min and then cooled at 5°/min to −90°C, held at this temperature for 10 min, and then heated at 5°C/min to 70°C. Scans were calibrated to 1 mg sample weight.

Thin-layer chromatography (TLC). Lipids were separated on silica gel TLC plates (Silica gel 60, 250 μm; E. Merck, Darmstadt, Germany). The mobile phase used was hexane/diethyl ether/glacial acetic acid (80:20:1, by vol; Ref. 10). This allowed separation of total lipids into 6–8 distinct bands with triacylglycerols (TAG) migrating at $R_i = 0.6$. Iodine vapor was used for visualization of lipid fractions. Lipid standards were obtained from Sigma (St. Louis, MO), Alltech Associates (Deerfield, IL), and Nu-Chek-Prep (Elysian, MN). Peanut, corn and olive oil were purchased locally.

Internal standards (IS). Margaric acid (17:0) was used as an IS for high-performance liquid chromatography (HPLC) and gas chromatography (GC). Total saponifiable lipids were quantified based on the IS added before saponification. To quantitate FFA, the IS was added to total lipids before HPLC injection. For TAG, IS was added after elution of TAG from the HPLC column. Different quantities of IS were added depending upon the lipid component being analyzed.

HPLC. Two silica-based C_{18} columns (15 cm, 5 μm particle size; Shodex/Waters, Milford, MA) in series and connected to a Waters HPLC instrument were used to separate TAG from FFA and fatty acid methyl esters (FAME). The mobile phase used in the isocratic mode was acetonitrile/isopropanol/methanol/hexane (8:5:3:1, by vol) at

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Abbreviations: DSC, differential scanning calorimetry; FAME, fatty acid methyl ester(s); FFA, free fatty acid(s); GC, gas chromatography; HPLC, high-performance liquid chromatography; ME, methyl esters; IS, internal standard; MSD, mass selective detector; TAG, triacylglycerol(s), triglyceride(s); TCK, *Tilletia controversa* Kühn; TCT, *T. tritici* Tul.; TLC, thin-layer chromatography; TSL, totalsaponifiable lipids.

room temperature. All solvents were HPLC-grade and from either Burdick and Jackson (Muskegon, MI) or Fisher (Fair Lawn, NJ). A flow program was used to optimize separation, *i.e.*, 1.5 mL/min initially, decreasing to 1.2 mL/min at 0.07 mL/min, then increasing to 2.0 mL/min at 0.07 mL/min. Eluting lipids were monitored by ultraviolet detector (Perkin-Elmer, Mountain View, CA) at 220 nm. The injection volume for optimum peak separation of individual TAG was 20 to 40 μg/μL lipid in 10 μL hexane. For quantitation of lipid classes, up to 400 μg of lipid was injected. Larger amounts of lipid caused retention time alterations and poor resolution of peaks, but allowed separation of FFA from TAG.

Derivatization of lipids. FAME from total lipids and TAG were made by heating with methanolic KOH. After neutralization and extraction with hexane, the dried extracts were heated with 14% (wt/vol) boron trifluoride (Alltech Associates) to ensure complete methylation (11). FAME were extracted and stored in isooctane. The FFA fraction collected by HPLC was directly methylated with boron trifluoride (11).

Identification of FAME. FAME were identified and quantitated using a Hewlett-Packard 5880 gas chromatograph equipped with a flame-ionization detector and capillary injection system (Palo Alto, CA; Model 18835B, HP). A fused silica capillary column (30 m × 0.25 mm) coated with Stabilwax CW20M (df = 0.25 μm; Restek, Bellefonte, PA) was used. Detector and injector temperatures were 280 and 260°C, respectively. Initial oven temperature was 60°C and was increased to 110°C at 25°C/min. The temperature program was slowed to 5°C/min until 250°C and held for 10 min. Helium was the carrier gas, and splitless injection was used.

All FAME were identified by retention time comparisons and mass spectrometry. An HP 5790 gas chromatograph was used and was coupled to an HP 5970 quadrapole-based mass selective detector (MSD). A fused silica capillary column (25 m × 0.2 μm) coated with dimethyl silicone (df = 0.1 μm; HP-1) was used to separate FAME before introduction into the MSD through a direct capillary interface. Helium was used as carrier gas (1 mL/min) with splitless injection. The oven temperature was initially held at 60°C for 1 min, then increased to 140°C at 25°C/min, then to 220°C at 3°C/min, and finally to 280°C at 5°C/min. Injector temperature was 260°C and the MSD interface was held at 280°C. The MSD was operated mainly in the scan mode with the selected ion monitoring mode reserved to measure trace amounts of FAME. Ionization voltage was 70 eV with the ion source being operated at the fixed nominal temperature of 250°C. Identification of peaks was based on comparison with known spectra and retention times.

RESULTS AND DISCUSSION

Past research on teliospores of TCT and TCK indicated that FFA were the major lipid components (4,5). Examination of teliospores by light (6,12) and electron microscopy (13) showed numerous lipid droplets within the endospore. DSC analysis of whole teliospores and of lipid extracts from teliospores showed an endothermic peak at −25 to −15°C and an exothermic peak at −40 to −45°C (Fig. 1). This peak was in the temperature range expected for unsaturated TAG rather than FFA (14).

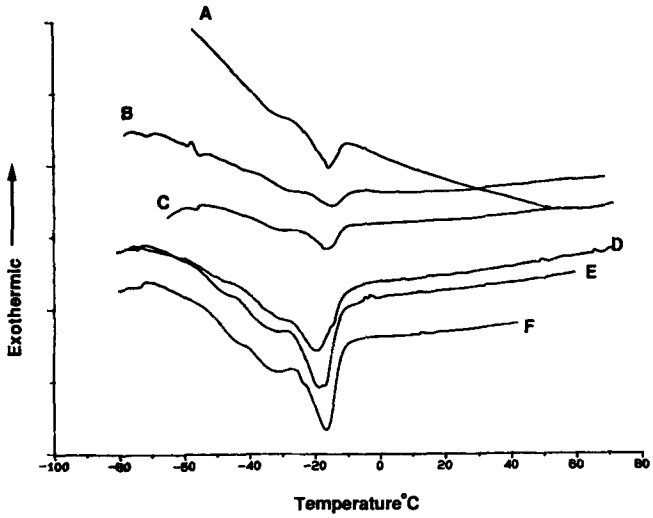

FIG. 1. Differential scanning calorimetry thermograms of whole teliospores of *Tilletia controversa* (A), *T. tritici* (TCT) 19 (B), TCT 4 (C), and lipid extracts from same (D), (E) and (F), respectively. For experimental details, see Materials and Methods.

TLC analysis of lipids (Fig. 2) showed that the FFA content of TCK and TCT was dependent upon the method of lipid extraction. Fishwick and Wright (9) showed that use of boiling isopropanol during extraction prevented enzymatic lipid degradation. The lipid profiles of TCK and TCT were altered dramatically when endogenous lipases were inactivated by boiling isopropanol. It is clear that when teliospores are ground in water or exposed to water (6), a substantial amount of FFA appear. Additionally, when teliospore homogenates were left in water for longer periods of time, the FFA spot in TLC increased in density (not shown), most likely due to the hydrolysis of TAG

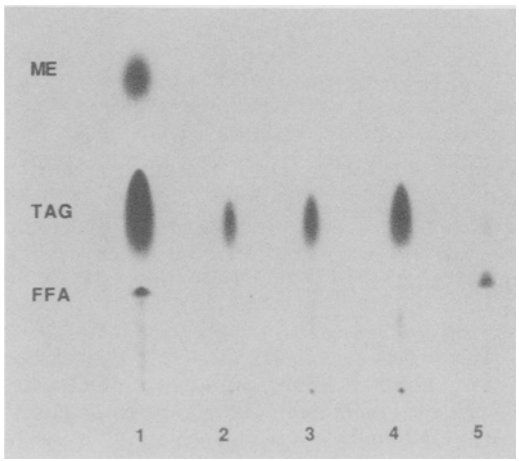

FIG. 2. Thin-layer chromatography of total lipids extracted from teliospores of *Tilletia* spp. Lane 1, standards; lane 2, *T. controversa* (TCK), homogenized in hot isopropanol; lane 3, *T. tritici* (TCT) 19, homogenized in hot isopropanol; lane 4, TCT 4, homogenized in hot isopropanol; lane 5, TCK, homogenized in water. Hexane/diethyl ether/glacialacetic acid (80:20:1, by vol) was used as mobile phase. FFA, free fatty acids; TAG, triacylglycerols; ME, methyl esters (FAME).

TABLE 1

Fatty Acid Profiles of Lipid Classes of *Tilletia tritici* and *T. controversa*

Organism	Lipid class[b]	% Total lipids (SEM)	Relative % FAME[a]								
			14:0	16:0	16:1	18:0	18:1[c]	18:2	24:0	24:1	24:2
Tilletia tritici (T19)	FFA	1.0 (0.1)	<0.5	33.0	7.8	8.7	13.1	39.1	<0.5	6.1	<0.5
	TAG	64.8 (5.9)	<0.5	13.3	1.1	1.4	11.5	61.5	1.6	5.2	0.7
	TSL	92.6 (3.9)	<0.5	13.0	1.1	1.1	11.3	61.3	1.6	5.3	0.7
T. tritici (T4)	FFA	2.0 (0.1)	2.0	21.4	5.6	11.6	12.5	44.4	<0.5	2.1	<0.5
	TAG	65.6 (3.8)	0.8	15.8	3.7	1.8	10.1	60.4	0.8	6.0	<0.5
	TSL	92.4 (4.6)	0.7	14.5	3.6	0.9	9.8	59.3	0.8	6.8	<0.5
T. controversa	FFA	1.1 (0.0)	<0.5	26.1	3.8	5.8	14.8	42.8	2.6	5.7	<0.5
	TAG	78.5 (0.7)	<0.5	14.2	2.4	1.0	8.4	64.6	0.8	5.2	0.6
	TSL	87.4 (1.4)	<0.5	13.9	2.3	0.8	8.3	64.5	0.9	5.5	0.7

[a]Other fatty acids found at less than 0.5% include 20:0, 20:1, 20:2, 22:0, 22:1, 22:2, and 18:3; FAME, fatty acid methyl esters.
[b]Abbreviations: FFA, free fatty acids; TAG, triacylglycerols; TSL, total saponifiable lipids.
[c]Δ9 and Δ11 isomers.

and/or phospholipids by lipases. The activities of endogenous lipases are actually the basis of a rapid viability test used for teliospores of TCK (15).

Because DSC and TLC indicated that TAG were the major lipid class, we quantitated the major lipid components by use of an internal standard. When the internal standard was injected into the HPLC, it eluted within the first 4 min along with all other FFA with a recovery exceeding 90%. Under the HPLC conditions used, all pure FFA and FAME eluted within 4 min.

The FFA content of TCK or TCT was determined to be 1–5% of the total lipid extracted (Table 1). Most fungal spores and mycelia have FFA contents within this range (16). The total lipid content of teliospores was found to be 30–35% (by weight) of which over 80% was saponifiable. The fatty acid compositions of the lipid components are given in Table 1. The FAME profile of TCK is similar to that reported by Trione and Ching (5), although the amount of FFA found in the current study (Table 1) was ten times less. Palmitic acid was reported to be the most abundant FFA found in dormant teliospores of TCT (17). This is in agreement with the data shown in Table 1 for TCT races 19 and 4. However, the amount of FFA found in the current study is again about ten times less than that found by others (4).

Total lipid and TAG FAME profiles were very similar (Table 1). Linoleic acid was the most common fatty acid, followed by palmitic acid. The FAME profile of FFA showed more of the saturated and monounsaturated fatty acids. The low amounts of FFA found suggest that there was little lipase activity during extraction. The presence of large amounts of linoleic acid in TAG of TCT and TCK might be expected, considering the fact that these teliospores must survive cold winter conditions (1,2), and crystallization could rupture storage vesicles. Additionally, TCK must maintain fluid lipid reserves, since the germination temperature is 4–7°C, and the fungus uses lipids as an energy source during germination. Finally, the host lipid composition may affect the composition of the lipids stored during sporogenesis of TCK and TCT.

Lipids, in particular FFA and oxidation products of FFA, have been suggested as the cause of dormancy of TCK (6). Earlier findings had indicated that lipids of TCT and TCK were composed mainly of FFA (4,5). When techniques that reduced degradation of TAG to FFA were employed, we found that 60–80% of the lipid component of *Tilletia* spp. was TAG. The amount of FFA in teliospores of TCT and TCK is closer to the amount of FFA found in other biological systems (*e.g.*, less than 5%). The remaining lipid components of teliospores are probably sterols and small amounts of phospholipids (16). These lipid components may play a more direct role in germination than the neutral lipids examined here. Based on these findings, the role of lipids as inhibitors of germination should be reevaluated.

ACKNOWLEDGMENTS

We thank Blair Goates for providing bunted wheat spikelets and advice. David Ruch is thanked for isolating teliospores.

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CORRECTIONS

The Excretion and Characterization of Intravenously Administered Olestra
R.J. Jandacek and B.N. Holcombe
Lipids 26, 754–758 (1991)

In this paper, the panels of Figure 1 (p. 757) were inadvertently transposed; 1a is the bottom panel, 1b is the top panel. The legend should read:

FIG. 1. Bottom (1a). Two-dimensional TLC autoradiogram of the [^{14}C]olestra used in Studies 2 and 3. A denotes the octaester; B, hepta- and hexaesters; C, unknown species. Top (1b). Two-dimensional TLC autoradiogram of the chloroform-soluble ^{14}C extracted from bile in Study 3. A and B are as in 1a; traces of radioactivity in area D may reflect lower esters.

Characterization of Triacylglycerols in the Seeds of *Aquilegia vulgaris* by Chromatographic and Mass Spectrometric Methods
Mustafa Demirbüker, Lars G. Blomberg, N. Urban Olsson, Magnus Bergqvist, Bengt G. Herslöf and Fernando Alvarado Jacobs
Lipids 27, 436–441 (1992)

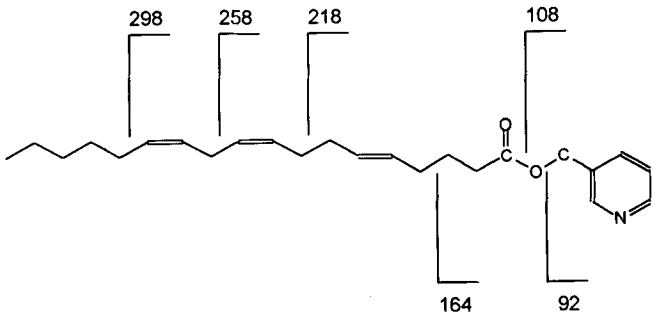
In this paper, fatty acids were numbered from the methyl terminus rather than starting from the carboxyl group. To translate the n-x terminology used into internationally accepted n nomenclature, the following changes are required:

Page 436, sixth line of Introduction section: "Columbinic acid (Cb), 6*c*,9*c*,13*t*-18:3" should read "5*t*,9*c*,12*c*-18:3 . . ."

Page 436, first line of Abbreviations section: ". . .9*c*,13*t*-18:2 . . ." should read ". . . 5*t*,9*c*-18:2 . . ."

Page 439, Table 1: "7*c*-18:1" should read "11*c*-18:1"; "11*c*-20:1" should read "9*c*-20:1"; "9*c*,13*t*-18:2" should read "5*t*,9*c*-18:2"; "6*c*,9*c*-18:2" should read "9*c*,12*c*-18:2"; "6*c*,9*c*,13*t*-18:3" should read "5*t*,9*c*,12*c*-18:3"; "3*c*,6*c*,9*c*-18:3" should read "9*c*,12*c*,15*c*-18:3"; "3*c*,6*c*,9*c*,12*c*-18:4" should read "6*c*,9*c*,12*c*,15*c*-18:4." The fatty acid nomenclature used throughout in the right column on page 439 should be changed accordingly.

Page 439, Scheme 1 should be:



The Editor-in-Chief wishes to thank Drs. Klaus Vosmann and Kurt Aitzetmüller, Institute for Chemistry and Physics of Lipids, Münster, Germany, for calling these errors to his attention.

Nonessential Fatty Acids in Formula Fat Blends Influence Essential Fatty Acid Metabolism and Composition in Plasma and Organ Lipid Classes in Piglets
Katharine M. Wall, Deborah Diersen-Schade and Sheila M. Innis
Lipids 27, 1024–1031 (1992)

The following changes should be made:

In Table 1 (p. 1025), the fatty acids listed as "20:0 + 14:0" should be "12:0 + 14:0"; the fatty acid composition of the diet is explained correctly in the text.

The second error occurred in the description of the homogenizing buffer used for brain and liver samples (p. 1025). This buffer contained 500 ng/mL leupeptin and 2 ng/mL aprotinen, not 500 mg/mL leupeptin and 2 μg/mL aprotinen.

Influence of Dietary Egg and Soybean Phospholipids and Triacylglycerols on Human Serum Lipoproteins
Barbara C. O'Brien and Verona Gale Andrews
Lipids 28, 7–12 (1993)

In Table 2 (p. 10), the values in the last two columns for HDL_2 PL should read 0.80 ± 0.19^c (instead of 0.36 ± 0.08) for SP treatment and 0.63 ± 0.15^b (instead of 0.35 ± 0.09) for SPTG treatment.

ARTICLE

Agonist-Induced Lipoxin A_4 Generation: Detection by a Novel Lipoxin A_4-ELISA

Bruce D. Levy[a,b], Sherry Bertram[c], H.H. Tai[d], Elliot Israel[b], Andrew Fischer[b], Jeffrey M. Drazen[b] and Charles N. Serhan[a,*]

[a]Hematology-Oncology Division, Department of Medicine, Brigham and Women's Hospital and [b]Combined Program in Pulmonary and Critical Care Medicine, Departments of Medicine, Brigham and Women's and Beth Israel Hospitals, Harvard Medical School, Boston, Massachusetts 02115, [c]ELISA Technologies, A Division of Neogen Corporation, Lexington, Kentucky 40509 and [d]Division of Medicinal Chemistry and Pharmaceuticals, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082

Lipoxin A_4 (LXA_4) possesses potent bioactions. To facilitate its detection, an enzyme-linked immunosorbent assay (ELISA) was developed that proved sensitive and selective. Quantitation by ELISA of LXA_4 generated from cellular sources strongly correlated ($r = 0.99$) with values obtained by high-pressure liquid chromatography (HPLC). We used this LXA_4-ELISA to examine parameters influencing LXA_4 generation from endogenous substrates during human platelet-neutrophil (PLT-PMN) interactions *in vitro*. Agonist-induced LXA_4 production was clearly evident at a PLT-PMN ratio of 10:1, and recombinant human granulocyte/monocyte colony stimulating factor-priming of PMN augmented LXA_4 generation 5–6 fold. The chemotactic peptide formylmethionyl-leucyl-phenylalanine, platelet-derived growth factor and arachidonic acid (20:4n-6) each stimulated formation of immunoreactive LXA_4 ($iLXA_4$) in these co-incubations. The presence of $iLXA_4$ was also evaluated *in vivo* in aspirin-sensitive asthmatic patients who, in a randomized, double-blind crossover design, underwent nasal lavage after they each ingested a predetermined threshold dose of aspirin or placebo. Aspirin challenge provoked statistically significant increases in $iLXA_4$ in each patient ($P < 0.005$). These results validate the use of a solid-phase ELISA for detection of LXA_4. Furthermore, the use of this ELISA has allowed the first documentation of $iLXA_4$ formation in human subjects with aspirin-sensitive asthma following specific antigenic challenge.

***Lipids* 28, 1047–1053 (1993).**

Lipoxins (LX) are bioactive eicosanoids generated by the interaction of individual lipoxygenases (LO) (reviewed in Ref. 1). These compounds can be produced by single cell types from endogenous sources of 20:4n-6 (2) or by cell-cell interactions that initiate transcellular pathways for their biosynthesis (3). Multiple biosynthetic routes can lead to LX generation (1). In human cell types, two main pathways have been demonstrated: one involves the transcellular metabolism of (15*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid (15-HETE) by 5-LO (4), and the other utilizes (5*S*)-(*E*)-5(6)-oxido-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid (LTA_4) that is converted by either 12-LO (3) or 15-LO (5,6). Using physical methods, (5*S*),(6*R*),(15*S*)-trihydroxy-(7*E*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid (LXA_4) has been identified *in vivo* in two different pathophysiologic settings—after percutaneous transluminal coronary angioplasty (PTCA) (7) and in bronchoalveolar lavage samples from patients with respiratory diseases (8).

LX possess biological activity in subnanomolar quantities in experimental models (reviewed in Ref. 1). Evidence is accumulating that LXA_4 may play a role as an endogenous regulator of inflammation (4,9–11). As is the case with other bioactive eicosanoids, it is critical to have a rapid and sensitive means for the detection of LXA_4 to assess the role of LXs in both physiological and pathophysiological events. To this end, we developed a solid-phase, enzyme-linked immunosorbent assay (ELISA), and here report its validation and utility in detecting LXA_4 in biological fluids both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials. Formylmethionyl-leucyl-phenylalanine (fMLP), A23187, nordihydroguaiaretic acid (NDGA) and natural human platelet-derived growth factor (PDGF)-AB were purchased from Sigma Chemical Co. (St. Louis, MO); thrombin and leukocyte separation media were from Enzyme Research Laboratories (South Bend, IN). Recombinant human granulocyte/monocyte colony stimulating factor (GM-CSFrh) was purchased from Boehringer Mannheim (Indianapolis, IN). Synthetic LXA_4 (molecular weight 352.5 and extinction coefficient 50,000) was obtained from Cascade Biochem Limited (Reading, Berkshire, England), (5*S*),(14*R*),(15*S*)-trihydroxy-(6*E*,8*Z*,10*E*,12*E*)-eicosatetraenoic acid (LXB_4) was from Biomol Research Laboratories (Plymouth Meeting, PA), and (5*S*),(6*R*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid [(5*S*),(6*R*)-DiHETE] was from Cayman Chemical Co. (Ann Arbor, MI). High-pressure liquid chromatography (HPLC) grade solvents were purchased from Doe and Ingalls (Medford, MA), methyl formate was from Eastman Kodak Co. (Rochester, NY) and Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA).

Cell suspensions. Using acid citrate dextrose as an anticoagulant, fresh peripheral blood was obtained by

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Abbreviations: ASA, aspirin; (5*S*),(6*R*)-DiHETE, (5*S*),(6*R*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid; ED, electrochemical detection; ELISA, enzyme-linked immunosorbent assay; fMLP, formylmethionyl-leucyl-phenylalanine; GC/MS, gas chromatography/mass spectrometry; GM-CSFrh, recombinant human granulocyte/monocyte colony stimulating factor; 5-HETE, (5*S*)-hydroxy-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid; 12-HETE, (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 15-HETE, (15*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid; HPLC, high-pressure liquid chromatography; $iLXA_4$, immunoreactive LXA_4; LX, lipoxins; LO, lipoxygenases; LTA_4 (leukotriene A_4), (5*S*)-(*E*)-5(6)-oxido-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid; LTB_4, (5*S*),(12*R*)-dihydroxy-(6*Z*,8*E*,10*E*,14*Z*)-eicosatetraenoic acid; LTC_4, (5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid; LXA_4, (5*S*),(6*R*),(15*S*)-trihydroxy-(7*E*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid; LXB_4, (5*S*),(14*R*),(15*S*)-trihydroxy-(6*E*,8*Z*,10*E*,12*E*)-eicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; PDGF, platelet-derived growth factor; PLT, platelet; PMN, neutrophil; RP-HPLC, reverse-phase high-pressure liquid chromatography; UV, ultraviolet.

venipuncture from healthy donors who had not taken aspirin or other medication for at least seven days. After the platelet-rich plasma was obtained, platelets (PLT) were washed in *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid-Tyrode buffer (pH 7.4) in the presence of ethylenediaminetetraacetic acid disodium dihydrate (EDTA; 7 mM), and then enumerated using a Coulter Counter (model ZBI; Coulter Electronics, Inc., Hialeah, FL). Neutrophils (PMN) were isolated from the remaining blood by Ficoll-Hypaque gradient centrifugation and dextran sedimentation (3). Suspensions contained 98 ± 1% PMN when enumerated by light microscopy.

PLT-PMN Incubations. Freshly isolated PMN were incubated in Dulbecco's phosphate-buffered saline containing both $CaCl_2$ (0.6 mM) and $MgCl_2$ (1.0 mM; pH 7.4) for 90 min at 37°C in the presence or absence of GM-CSFrh (200 pM). Aliquots (0.5 mL) of each cell suspension (8–30 × 10^6 PMN/mL) were combined with aliquots (0.5 mL) of isolated PLT suspensions with the cell ratio adjusted from 0:1 to 100:1 (PLT-PMN). PMN and PLT were warmed (5 min, 37°C) before exposure to soluble stimuli (20 min, 37°C). Incubations were stopped by the addition of 1 vol of 0.05 M Tris-HCl (pH 8.0) and vigorous mixing. Materials were kept at −30°C for at least 30 min prior to analysis to facilitate protein precipitation, as well as to retard autooxidation.

Nasal lavage fluid. After written informed consent to a protocol approved by the Beth Israel Hospital Committee on Clinical Investigations, subjects with aspirin-sensitive asthma were identified, and a threshold dose of aspirin (ASA) was determined that would elicit, 1–3 h after ingestion, a ≥ 15% decline in the FEV_1; in these subjects bronchospasm was often accompanied by oculo-nasal symptoms. In a randomized, double-blind, crossover design, nasal lavages were performed as previously described (12) with 0.9% saline (8 mL) two and three hours after ingestion of either placebo (lactose) or the threshold dose of aspirin (60–100 mg). Patients were 32–42-years-old, without a history of recent illness or tobacco use for ≥10 yr, and had normal baseline spirometry with an FEV_1 that decreased 16–25% with ASA challenge. Subjects were allowed to use inhaled steroids; however, theophylline was discontinued for 48 h prior to provocation and inhaled β-agonists and nasal steroids were withheld for at least 8 h prior to challenge. All lavage fluids were stored at −70°C.

LXA_4-ELISA. LXA_4 antiserum was produced in a similar manner to that described for TxB_2 antiserum (13). Briefly, LXA_4 was conjugated to keyhole limpets hemocyanin through succinimide ester of LXA_4. Rabbits were immunized initially with 100 μg of LXA_4-hemocyanin conjugate per rabbit. Booster injections were carried out on a monthly basis with half of the initial conjugate. The rabbits were bled through ear veins and the antisera were collected by centrifugation of the blood and stored at −20°C. The antiserum was diluted (1:200) in ELISA buffer to a concentration optimized for assay conditions (*vide infra*). LXA_4 was labeled with horseradish peroxidase through succinimide ester of LXA_4 as described above. The enzyme-labeled LXA_4 and the antisera were used to develop an ELISA for LXA_4.

The ELISA buffer containing 0.1% bovine serum albumin and 0.9% NaCl in 0.1 M potassium phosphate buffer, pH 7.4, was used for diluting antisera, standard or sample, and enzyme conjugate. Prior to assay, methanol and ethanol were eliminated from all samples, as traces of organic solvent interfered with ligand-antibody binding. The 96-well plate was precoated with 1 μg of affinity-purified goat anti-rabbit IgG per well. The assay was initiated by adding appropriately diluted antisera (50 μL), LXA_4 standard or samples (50 μL) and enzyme-labeled LXA_4 (50 μL) into each well. Plates were gently shaken for 1 h at room temperature. After three washings with wash buffer (0.01 M potassium phosphate, pH 7.5 containing 0.05% Tween 20), the enzyme reaction was carried out by adding 150 μL of K-Blue substrate at room temperature for approximately 15 min and stopped by the addition of 100 μL of 1N H_2SO_4. The absorbance at 450 nm was read using a Dynatech Plate Reader (model No. MR 700; Alexandria, VA). Serial dilutions of unlabeled synthetic LXA_4 (0.20–2.00 ng LXA_4/mL) were assayed and included within each 96-well plate to generate calibration curves for quantitation of immunoreactive LXA_4 ($iLXA_4$) (ng/mL) present in samples; all samples were assayed in duplicate.

Quantitation of LXA_4 by electrochemical detection-ultraviolet (ED-UV) reverse phase (RP) HPLC. Prostaglandin B_2 (25 ng) was added, as an internal standard, to materials to be analyzed by HPLC. Samples were extracted and eluted from Sep-Pak C_{18} cartridges, and methyl formate fractions were concentrated under a stream of N_2 as in (3). This RP-HPLC system was equipped with both a Lambda-Max UV detector, model 481, and an on-line Electro Chemical detector (ED), model M 460, operated with a $Ag/AgCl_2$ reference electrode (Waters Associates) that permitted detection of LXs in the picogram range (3). The electrode potential was set at 1.345 V, and the UV detector was set at 300 nm to monitor the LX tetraene chromophore. LXA_4 was identified by comparison of its retention time with those of synthetic and authentic LXA_4 standards, and quantities were determined by comparison to areas beneath the peaks obtained for UV-calibrated authentic LXA_4 after adjustment for recovery of internal standard.

All data are expressed as the mean ± SE of the mean (SEM). The Student's *t*-test was used for statistical calculations on the naturally paired *in vitro* cell suspensions reported later and the self-paired patient samples in Table 1. *P* values <0.05 were considered significant. All analyses were performed on a Macintosh computer (Apple Computer, Inc., Cupertino, CA) with the use of Statworks (Cricket Software, Inc., Philadelphia, PA).

RESULTS

ELISA sensitivity and selectivity. After conditions for the enzyme immunoassay of LXA_4 were optimized, calibrated dose-response curves were obtained using serial dilutions of competing unlabeled synthetic LXA_4 (Fig. 1A). Inhibition of 50% of the maximal binding (IC_{50}) observed with enzyme-labeled LXA_4 occurred with 0.45 pmol LXA_4/mL, and the minimum detectable amount (80% of maximal binding) was 90.8 fmol LXA_4/mL. This IC_{50} is comparable to those obtained by ELISA for other eicosanoids: 0.4 pmol (14) and 0.2 pmol/mL for (5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid (LTC_4) (15), 0.49 pmol/mL for 6-keto-$PGF_{1\alpha}$ (16), 1 pmol/mL for 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (17) and

0.54 (18) and 0.4 pmol/mL for TxB_2 (15). Crossreactivity of the antibody for related compounds was 0.1% for 15-HETE, and <0.1% for (5*S*)-hydroxy-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (5-HETE), (12*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE), (5*S*),(12*R*)-dihydroxy-(6*Z*,8*E*,10*E*,14*Z*)-eicosatetraenoic acid (LTB_4), LTC_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycyl-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid (LTD_4), (5*S*)-hydroxy-6(*R*)-*S*-cysteinyl-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid (LTE_4) and 20:4n-6 (Fig. 1A, insert). Further experiments on the stereoselectivity of antibody binding employed lipoxygenase products with structures identical to portions of native LXA_4, namely (5*S*),(6*R*)-DiHETE (C-1 to C-6) and LXB_4 (C-1 to C-5 and C-15 to C-20). Crossreactivity for these closely related eicosanoids was <5% for 5(*S*),6(*R*)-DiHETE and between 1–7.5% for LXB_4 (Fig. 1A). These results indicate that the antiserum is both sensitive and selective for LXA_4.

Co-incubations of autologous human PLT and PMN in the presence of stimuli have been demonstrated by both HPLC and gas chromatography/mass spectrometry (GC/MS) to generate LXA_4 from endogenous sources (3, 19). For purposes of direct comparison to prior results and to evaluate the ELISA's use in the determination of LXA_4 generation by cellular sources, GM-CSFrh-primed PMN (15×10^6) were co-incubated with PLT (150×10^6) in the presence of A23187 (10^{-7} M) and thrombin (1 U/mL). After the co-incubations, serial dilutions of the materials generated were performed, and the quantities of LXA_4 present were determined using both ELISA and ED-UV RP-HPLC. Figure 1B shows results representative for PLT-PMN co-incubations that gave a correlation coefficient of 0.99. Together, these results demonstrate that quantitation by ELISA of biologically-derived LXA_4 correlates with values obtained following HPLC. In addition, the quantities of $iLXA_4$ identified are in good agreement with those reported using HPLC-based quantitation (3,19), and indicate that lower cell numbers than those required for analysis by HPLC and GC/MS can be used in each incubation.

Characteristics of $iLXA_4$ generation during PLT-PMN interactions. We used this assay to examine factors regulating LXA_4 generation by suspensions of human PMN co-incubated with increasing numbers of autologous PLT (Fig. 2). As GM-CSFrh is known to increase arachidonic acid availability and LXA_4 generation (3), the impact of adding this cytokine on LXA_4 generation was examined at different ratios of PLT-PMN. At each cell ratio examined (Fig. 2), PMN primed with GM-CSFrh (200 pM) generated higher levels of $iLXA_4$ when exposed (20 min, 37°C) to A23187 (10^{-7} M) and thrombin (1 U/mL). As the PLT-PMN ratio was increased to 10:1, GM-CSFrh-primed PMN led to generation of 5–6 times more LXA_4 than activated PMN that were not primed. At higher PLT number, homotypic PLT-PLT interactions led to decreased LXA_4 generation. Also, in the presence of stimuli (A23187 or fMLP plus thrombin), neither PMN alone nor isolated PLT (data not shown) generated $iLXA_4$ in statistically significant amounts greater than incubations performed in the absence of stimuli. Primed PMN in the presence of stimuli, but without deliberate addition of PLT, generated nanomolar quantities (≈3 nM $iLXA_4$/incubation) of $iLXA_4$. Because limitations in the isolation of PMN resulted in ≈1–2 PLT per PMN contaminating

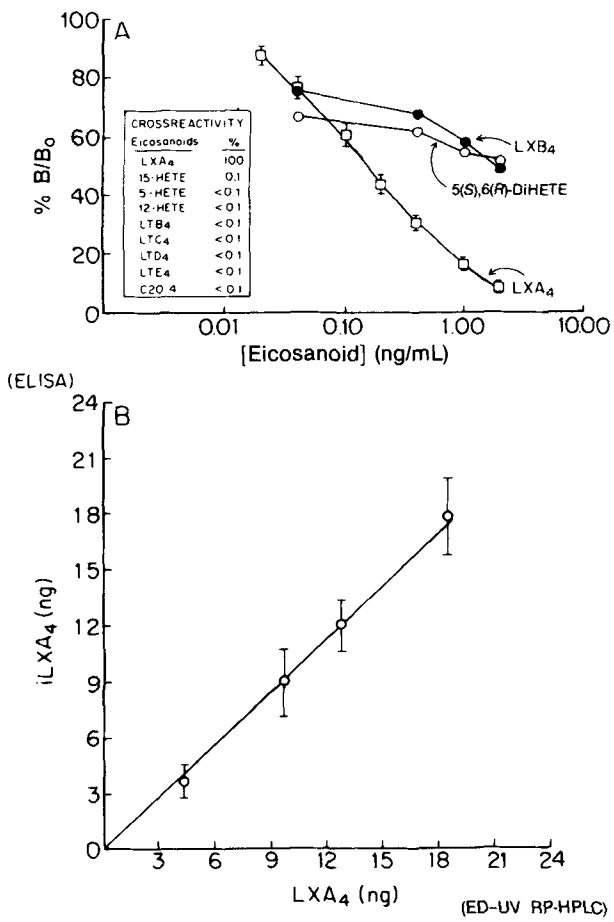

FIG. 1. Quantitation of lipoxin A_4 by enzyme-linked immunosorbent assay (ELISA): crossreactivity with related eicosanoids and correlation with high-pressure liquid chromatography (HPLC). A: Unlabeled (5*S*),(6*R*),(15*S*)-trihydroxy-(7*E*,9*E*,11*Z*,13*E*)-eicosatetraenoic acid (LXA_4) (0.02 to 2.00 ng/mL), (□) was added to a standard amount of enzyme-labeled LXA_4 and incubated (60 min, 23°C) with the antibody (n = 15, mean ± SEM). On the vertical axis, % B/B_0 represents the bound enzymatic activity in the presence (B) of unlabeled LXA_4 relative to its absence (B_0). To examine the antibody's stereoselectivity, 0.04 to 2.00 ng/mL of (5*S*),(6*R*)-dihydroxy-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid [(5*S*),(6*R*)-DiHETE] (○) and (5*S*),(14*R*),(15*S*)-trihydroxy-(6*E*,8*Z*,10*E*,12*E*)-eicosatetraenoic acid (LXB_4) (●) were added to enzyme-labeled LXA_4 and incubated in parallel (n = 3, mean). The insert reports the crossreactivity of related compounds when identical quantities of unlabeled material were added in place of LXA_4. B: Freshly isolated human neutrophils (15×10^6) were exposed to 200 pM recombinant human granulocyte/monocyte colony stimulating factor (90 min, 37°C) prior to incubation (20 min, 37°C) with autologous platelets (150×10^6) in the presence of A23187 (10^{-7} M) and thrombin (1 U/mL). Incubations were stopped with 1 vol of ice-cold, 0.05 M Tris-HCl (pH 8.0). Serial dilutions of the products generated were assayed by both ELISA and electrochemical detection-ultraviolet (ED-UV) reverse-phase (RP) HPLC. Prior to RP-HPLC, the materials were purified by extraction with Sep-Pak C_{18} cartridges in the presence of an internal standard (see Materials and Methods section). These results were plotted from a representative incubation (cells from two separate donors, four determinations for each concentration) for regression analysis (r = 0.99, y = 0.98x − 0.19). ELISA values represent the mean of two separate assays. $iLXA_4$, immunoreactive LXA_4.

these suspensions, we were unable to determine the amount of $iLXA_4$ produced by PMN alone. Intentional addition of PLT dramatically increased formation of $iLXA_4$ (Fig. 2) to levels with a range of bioactions

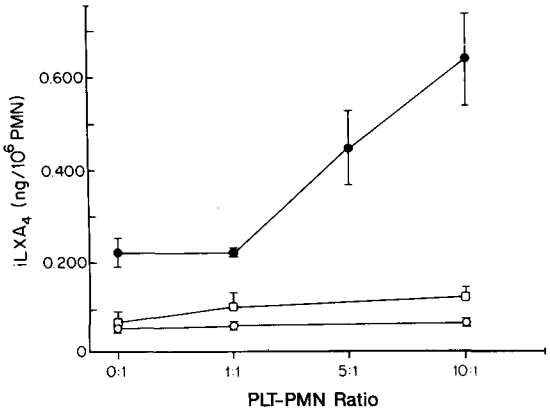

FIG. 2. Generation of iLXA$_4$ from endogenous sources during co-incubations of platelet-neutrophil (PLT-PMN): effect of cell ratio (PLT-PMN) and recombinant human granulocyte/monocyte colony stimulating factor (GM-CSFrh) priming of PMN. PMN (8–10 × 10^6) were incubated in the presence of GM-CSFrh (200 pM) for 90 min at 37°C, and then combined with autologous PLT. After warming (5 min, 37°C), cells were exposed to either A23187 (10^{-7} M) and thrombin (1 U/mL) (●) or vehicle control (○) for 20 min at 37°C. In parallel, PMN, not primed with GM-CSFrh, were co-incubated with PLT and exposed to the soluble stimuli (□). Incubations were stopped with 1 vol of cold 0.05 M Tris-HCl (pH 8.0). ELISA was performed without further purification of products, (n = 3 separate donors, mean ± SEM). Abbreviations as in Figure 1.

(reviewed in Ref. 1), including the stimulation of myelopoiesis (20). These findings are consistent with production of leukotriene A$_4$ by activated PMN that is released and transformed to LXA$_4$ by platelet 12-lipoxygenase (1). Additionally, stimulus-induced formation of iLXA$_4$ was maximal during co-incubations that were held motionless; shaking the co-incubations inhibited iLXA$_4$ formation (>83%, data not shown). This finding suggests that interference with PLT-PMN interactions impairs the transcellular exchanges of biosynthetic intermediates, such as LTA$_4$. Thus, taken together, these results obtained using independent methodologies both confirm and extend the findings that activated cytokine-primed PMN interacting with PLT have the potential to generate biologically relevant quantities of LXA$_4$ (3).

PDGF is also known to promote activation of PMN (21,22). To determine if this cytokine also stimulates LXA$_4$ generation, GM-CSFrh-primed PMN were co-incubated with autologous PLT in the presence of PDGF and the chemotactic peptide, fMLP, which has been previously shown to activate PMN for transcellular biosynthetic generation of LXA$_4$ (3). Indeed, cells exposed to either fMLP (10^{-7} M) or PDGF-AB (10 ng/mL) generated more iLXA$_4$ from endogenous sources of substrates than from unstimulated cells (Fig. 3A). When exogenous arachidonic acid was added to these incubations, increases in iLXA$_4$ levels were obtained upon exposure to fMLP (410%, $P < 0.02$) or PDGF (381%, $P < 0.09$). Moreover, arachidonic acid itself may be a second messenger (23), as it is known to activate both PMN and PLT (24), and PLT can contribute their 20:4n-6 to be transformed by PMN in a bidirectional route to LXA$_4$ (3). In these experiments, exogenous 20:4n-6 also stimulated LXA$_4$ generation (Fig. 3A). Agonist-induced formation of iLXA$_4$ from endogenous sources of sub-

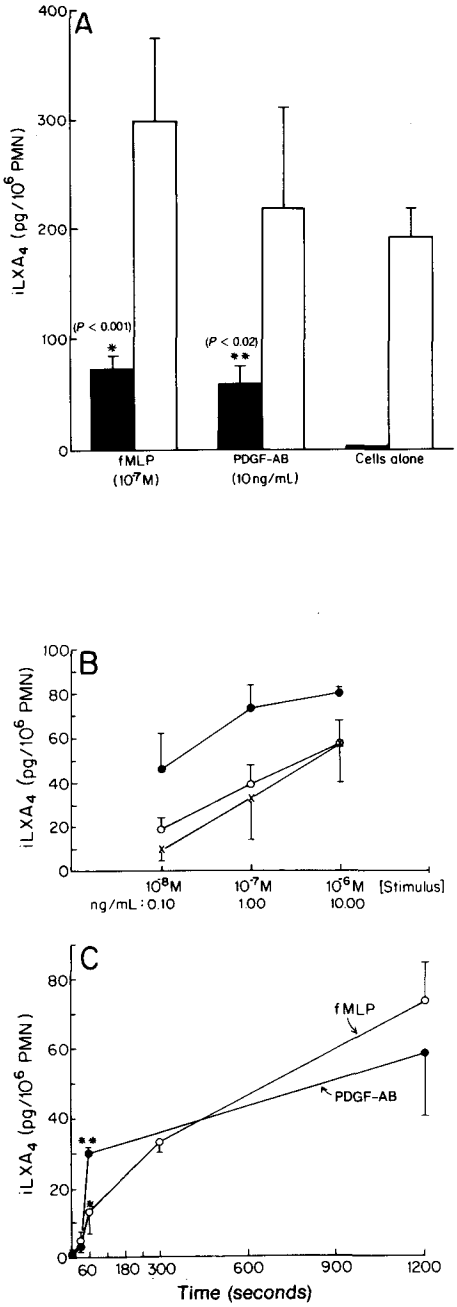

FIG. 3. Generation of iLXA$_4$ from endogenous and exogenous arachidonic acid during co-incubations of GM-CSFrh-primed PMN with autologous PLT. PMN (10 × 10^6) were exposed (90 min, 37°C) to 200 pM GM-CSFrh and then incubated (20 min, 37°C) with autologous PLT (100 × 10^6) in the presence of stimuli. Incubations were terminated and assayed as described in Materials and Methods. Values are expressed in pg/10^6 PMN (mean ± SEM, n = 3) minus values obtained without stimuli present in parallel incubations. A: Exogenous arachidonic acid. Parallel incubations were performed in the presence (open bars) or absence (solid bars) of exogenous arachidonic acid (10 μM) during simultaneous exposure to either formylmethionyl-leucine-phenylalanine (fMLP) (10^{-7} M), PDGF-AB (10 ng/mL), or vehicle control, (*, $P < 0.001$; ** $P < 0.02$). B: Concentration response. Incubations were carried out in the presence of PDGF-AB reported in ng/mL (x), fMLP alone (●) or fMLP and thrombin (1 U/mL) (○). C: Time course. Incubations were terminated at the indicated time points after exposure to either 10^{-7} M fMLP (○) or 10 ng/mL PDGF-AB (●) (* $P < 0.05$; ** $P < 0.01$). Abbreviations as in Figures 1 and 2.

TABLE 1

Elevated $iLXA_4$ in Nasal Lavage Fluid After Aspirin Challenge in Aspirin-Sensitive Asthmatics[a]

Subject	Determinations	Placebo (pg $iLXA_4$/mL)	Aspirin (pg $iLXA_4$/mL)
1	2	Not detected	47.5 ± 3.5
2	8	61.0 ± 13.8[b]	318.9 ± 8.0 (523%)[b]
3	4	Not detected	47.7 ± 6.3
4	4	Not detected	41.7 ± 3.9
5	2	47.5 ± 0.5[b]	312.5 ± 9.5 (658%)[b]

[a]Subjects were lavaged with 0.9% saline 2 and 3 h after ingestion of either a placebo or a threshold dose of aspirin. Levels of $iLXA_4$ were determined by enzyme-linked immunosorbent assay (see Materials and Methods). Results represent the mean ± SEM of these two lavages. The percent increase in levels of $iLXA_4$ in lavage fluid after aspirin challenge compared with placebo is parenthetically displayed for patients 2 and 5. $iLXA_4$, immunoreactive (5*S*), (6*R*), (15*S*)-trihydroxy-(7*E*,9*E*,11*Z*,13*E*) eicosatetraenoic acid.
[b]$P < 0.005$.

strate proved to be both concentration- and time-dependent (Fig. 3B and C). PDGF (10 ng/mL) and fMLP (10^{-6} M) plus thrombin (1 U/mL) led to approximately equivalent levels of $iLXA_4$ (Fig. 3B). In addition, the divalent cation ionophore A23187 led to a concentration-dependent (10^{-8}–10^{-5} M) increase in $iLXA_4$ (0.004–2.340 ng $iLXA_4/10^6$ PMN). With each stimulus examined, $iLXA_4$ generation was rapid in onset and statistically significant ($P < 0.05$) within 60 s (Fig. 3C). Exposure to NDGA (100 μM), a nonselective LO inhibitor that blocks 5-, 12- and 15-LO, led to complete inhibition of $iLXA_4$ formation during co-incubations (cells from two separate donors with four determinations). Together, these findings confirm that $iLXA_4$ is derived from arachidonic acid and demonstrate that its formation is triggered by either fMLP or PDGF.

Aspirin challenge in sensitive asthmatics provokes $iLXA_4$ formation. When challenged with a dose of ASA sufficient to lead to airflow obstruction, ASA-sensitive asthmatics generate cysteinylpeptide leukotrienes in amounts substantially higher [in nasal lavage (25) and urine (26)] than ASA-insensitive individuals. Since LXA_4 could also be formed with 5-LO activation (1,8), we next evaluated LXA_4 formation in ASA-sensitive patients using the ELISA to determine if LXA_4 is generated *in vivo* after challenge. In a randomized, double-blind, crossover design, subjects were lavaged with normal saline after ingestion of either placebo or a predetermined threshold dose of ASA (see Materials and Methods section). Each subject thus served as their own "control." Quantities of LXA_4 added to nasal lavage fluid ("spiking samples") were quantitatively recovered by ELISA ($r = 0.95$, $n = 3$), indicating the absence of significant amounts of interfering substances. After ingestion of placebo, patients were asymptomatic, and $iLXA_4$ was not detected in lavages from three of five patients (Table 1). In sharp contrast, following aspirin challenge, $iLXA_4$ was present in significant quantities in lavages obtained from each patient. In those individuals where $iLXA_4$ was found after placebo challenge (Nos. 2 and 4), 5–6-fold increments in $iLXA_4$ were obtained after challenge with ASA. Hence, the presence of $iLXA_4$ after placebo that is significantly elevated after challenge with ASA may reflect, in part, their heightened sensitivity. These results demonstrate appearance of $iLXA_4$ after a specific challenge.

DISCUSSION

To explore the role of LXA_4 in human pathobiology requires a method to reliably detect it in subnanomolar amounts *in vitro* and pathologically-derived materials. To this end, we report on the validation of an ELISA for LXA_4 and its utility in examining the formation and presence of this eicosanoid. The developed assay proved both sensitive and selective for synthetic LXA_4 (Fig. 1A) with characteristics comparable to ELISA tests constructed for other eicosanoids (14–18). Authentic LXA_4 production from cellular sources was confirmed by ED-UV RP-HPLC (Fig. 1B), and parameters for agonist-induced generation of LXA_4 from endogenous substrates *in vitro* were determined (Fig. 2). fMLP and PDGF each triggered concentration- and time-dependent formation of $iLXA_4$ from endogenous stores of 20:4n-6 (Fig. 3), and patients with ASA-sensitive asthma had $iLXA_4$ in nasal lavage after ASA provocation (Table 1). Given the ELISA's ability to detect both synthetic and authentic LXA_4 from cellular sources (Figs. 1–3) and pathologic fluids (Table 1), it appears well-suited for use in exploring this eicosanoid's role in physiologic and pathophysiologic processes.

Enzyme immunoassays have been developed for many other eicosanoids, such as prostaglandins, LTB_4 and LTC_4 (cf. Refs. 14 and 15). Their use minimizes the time and extensive sample preparation required for physical methods of detection, like HPLC and GC/MS. Because of this ELISA's sensitivity (90.8 fmol LXA_4/mL) and selectivity (Fig. 1), it also permits efficient utilization of material. Using this method, we were able to detect LXA_4 after ASA provocation in aliquots (50 μL) of nasal secretions from asthmatic patients. To ensure the accuracy of the ELISA, nonspecific binding was routinely assayed on the 96-well plates as an internal control. In addition, a clear correlation between ELISA and ED-UV RP-HPLC in the generation of LXA_4 (Fig. 1) indicated the presence of insignificant amounts of crossreacting compounds in this system, and the accurate detection of known quantities of LXA_4 added to nasal lavage fluid demonstrated the absence of interfering substances in this biological matrix (see Results section). Therefore, this ELISA proved to be a sensitive and reliable technique for the detection of LXA_4.

Enabled by this new method, human PLT and PMN interactions were examined as an established *in vitro* model for LXA_4 production in the presence of stimuli that activate cells by receptor-mediated signals. These cell types were selected because together they possess the three major mammalian LO (5-, 12- and 15-LO) and can be readily obtained as freshly isolated cell suspensions. Human platelets possess high levels of 12-LO, and leukocytes possess both 5- and 15-LO. Interactions between these enzymes with 20:4n-6 as the substrate give rise to LXs (1). The generation of LXA_4 during co-incubations of PLT and PMN in the presence of increased numbers of PLT or with GM-CSFrh-priming of PMN (Fig. 2) not only confirmed previous reports using these combined cell suspensions (3,19), but also demonstrates LXA_4

formation from endogenous sources when PMN and autologous PLT are exposed to stimuli. Agonist-induced generation of $iLXA_4$ was rapid and concentration-dependent (Fig. 3B and C) with the reactions producing levels detectable by ELISA in the pico- to nanomolar range. It is important to point out that these levels of LXA_4 were achieved without the addition of exogenous 20:4n-6. Pico- to nanomolar quantities of LXA_4 have been demonstrated to elicit a range of bioactions in experimental models, including arteriolar dilatation and antagonism of LTB_4-induced PMN activation (reviewed in Ref. 1). In addition, PDGF was recently demonstrated to lead to the accumulation of neointimal smooth muscle after balloon-catheter deendothelialization of rat carotid arteries (27). This potent mitogen for smooth muscle is found in PLT and can activate many cell types, including PMN, leading to a chemotactic response (21,22). Similar to PDGF, LXA_4 is released intravascularly after balloon angioplasty in human coronary arteries (7). PDGF increases 20:4n-6 availability (28) and, in the present experiments, at biologically relevant concentrations led to the formation of $iLXA_4$ (Fig. 3). Thus, our findings suggest a potential link in PDGF and LXA_4 generation. Cytokine-primed PMN and PLT are likely to interact at sites of inflammation or vascular damage; thus, stimulus-induced LXA_4 generation may be relevant in these processes.

In humans, LXA_4 has been demonstrated in bronchoalveolar lavage fluid from asthmatics using HPLC and GC/MS (8). Aspirin-sensitive asthmatics are a select group of patients characterized by bronchospasm after ingestion of ASA (29). This cyclooxygenase inhibitor leads to higher levels of LO-derived products, including LXA_4, *in vivo* after PTCA (7) and, *in vitro*, does not inhibit lipoxin generation (1). Compared with ASA-insensitive individuals, sensitive asthmatics often have nasal polyps, which, *in vitro*, generate LXA_4 when incubated in the presence of either LTA_4 or activated neutrophils (6). Moreover, PLTs from these patients demonstrate abnormal responsiveness upon exposure to ASA (30). Results in Table 1 clearly indicate the presence of LXA_4 in nasal lavages after ASA ingestion. Although the present data do not permit an evaluation of the cellular source of LXA_4, there are several cell types present in these patients, such as eosinophils, cytokine-primed PMN, PLT, mast cells and nasal polyps, which are known to generate LX either individually or by transcellular biosynthesis *in vitro* that could have contributed to LXA_4 generation (reviewed in Ref. 1). Enabled by the ELISA's sensitivity and accuracy, these findings are the first to demonstrate the appearance of LXA_4 after a specific provocation *in vivo*, and indicate a potential role for LXA_4 in the airway. Recently, LXA_4 administered by nebulizer to humans has been shown to attenuate LTC_4-induced bronchoconstriction in asthmatic patients (10). Also, LXA_4 is further metabolized by phorbol myristate acetate-treated HL-60 cells and human monocytes to several major products including 15-oxo-LXA_4, 13,14-dihydro-15-oxo-LXA_4 and 13,14-dihydro-LXA_4 *in vitro* (31). It is not presently known if these metabolites represent the major route of LX metabolism *in vivo* and whether this ELISA can also be used to detect the metabolites. Thus, the present results indicate that further investigation is warranted in evaluating the role of LXA_4 in ASA-sensitive asthma and airway disease.

In conclusion, we present evidence for a new, sensitive and selective ELISA for LXA_4 and demonstrate its utility to detect LXA_4 *in vitro* and in pathologic fluids. Moreover, the availability of this assay enabled the demonstration of PMN and platelet agonists, such as PDGF, in the generation of LXA_4 during cell-cell interactions *in vitro* and documentation of the appearance of LXA_4 in humans after given an inflammatory stimulus.

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Triacylglycerol Assembly from Binary Mixtures of Fatty Acids by *Apiotrichum curvatum*

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To observe how the stereospecific distribution of acyl groups in triglycerides is affected by the composition of fatty acids available for esterification, the oleaginous yeast *Apiotrichum curvatum* was grown on various binary mixtures of palmitic, stearic, oleic and linoleic acids as carbon sources, and the yeast triglycerides were analyzed. When oleic acid–linoleic acid mixtures in various ratios were used as substrates, the yeast grew well, and the composition of the intracellular triglycerides reflected the substrate composition, but more linoleate than oleate was deposited in the triglycerides. Oleate was favored over linoleate at the *sn*-2 position of the glycerol. With substrates containing palmitic and stearic acids, the yeast accumulated less oil, and incorporation of stearic acid into the triglycerides also was very limited. When mixtures of palmitic acid–oleic acid and palmitic acid–linoleic acid were used as substrates, the yeast triglyceride composition did not reflect that of the substrate, and the accumulation in the yeast of the unsaturated acid in the substrate was favored. Possibly, the yeast had more limited access to solid than to liquid substrates. For oleic acid–linoleic acid substrates, when the percentages of oleate and linoleate at the three glycerol positions were plotted *vs.* the percentage of these acyl groups in the total triglyceride, apparent linear relations were observed for most of the range, and the sums of the intercepts and slopes of the three lines for each acyl group were 0 and 3, respectively. Two mathematical models of triglyceride assembly are proposed, both of which fit the experimental data. One model assumes that for a certain proportion of the glycerol molecules, the acyl composition of the three *sn* positions is rigidly controlled independently of the substrate concentration. The other assumes that the various acyl groups are distributed on the three *sn* positions of glycerol with different affinities.

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Triglyceride structure affects the consistency, stability and nutritional value of fats and oils (1–3), but because of the complexity of triglyceride mixtures, a complete analysis of triglycerides is seldom achieved. Stereospecific analysis of the acyl composition on the three positions of glycerol has revealed that the distribution of acyl groups is not random (3,4). It has been suggested that the restraints manifest in the stereospecific distribution account for the triglyceride structure, but analyses of olive oil suggest that other restrictions on the distribution also occur (5). Various proposals have been made to account for the distribution of acyl groups on the positions of glycerol, especially in oilseed, where the triglyceride supposedly is simply deposited during seed development. The distribution should reflect the biosynthetic pathways resulting in triglycerides and the specificity of the enzymes involved in the various steps, but the process is complicated by changes with time in the proportions of the acyl groups being deposited and by exchange reactions among the various lipid classes involved in the biosynthesis (6). Studies with labeled precursors and intermediates have led to helpful insights into triglyceride synthesis, but such studies often are limited by the insolubility of lipid substrates in water and the nonphysiological conditions sometimes required for the experiments.

A number of generalizations can be made about the observed distribution of acyl groups on glycerol, and these have been reviewed by Litchfield (3). One of these generalizations notes that, for a group of individual plants or animals with different acyl group compositions, there are linear relations between the percentages of a particular acyl group on the three glycerol positions and the percentage of that acyl group in the whole triglyceride mixture. Such linear relations have been reported in corn (7), soybean (4,8), oat (8), cruciferae (9) and peanut (10,11) oils.

The oleaginous yeast *Apiotrichum curvatum* ATCC 20509 (formerly known as *Candida curvata D*) will accumulate oil when grown on sugar or fat substrates if nitrogen for growth is limiting (12). It also will grow on fatty acids with 14–20 carbon chain lengths and accumulate triglycerides, but it grows best on palmitic, oleic or linoleic acid (13). It tends to deposit the same acyl groups that are in its substrate with minor modifications. In the yeast triglycerides, oleate tends to be favored on the *sn*-2 position of the glycerol, and saturated fatty acids are strongly favored on the *sn*-1 and *sn*-3 positions.

The characteristics of this yeast make it possible to study the effect of a wide range of oleate/linoleate ratios on the stereospecific distribution in the triglycerides, and the results of such a study are reported in this paper. From a few simple assumptions about triglyceride assembly, two models were derived that account for many of the relations observed in yeast and oilseeds.

MATERIALS AND METHODS

Apiotrichum curvatum was maintained as refrigerated slant cultures on yeast extract/dextrose/peptone/agar (1:2:2:1.5, by wt) and transferred monthly. The constituents of the basal medium were (g/L): KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.0; asparagine, 0.8; $CaCl_2 \cdot 2H_2O$, 0.2; NaCl, 0.06; $FeCl_3 \cdot 6H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.002; $ZnSO_4 \cdot 7H_2O$, 0.001; thiamine-HCl, 0.001; and $CuSO_4 \cdot 5H_2O$, 0.0001 (12). The basal medium was adjusted to pH 5.5 and supplemented with 18 g/L of fatty acids purchased from Sigma Chemical Co. (St. Louis, MO). When linoleic acid was used as a carbon source, it was supplemented with 1,000 ppm of butylated hydroxyanisole to prevent oxidation during incubation.

A seed culture was prepared by inoculating about 5×10^5 cells from a slant culture into 100 mL of heat-

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sterilized basal medium with the fatty acids isolated from saponified corn oil as a carbon source. The culture was grown in 250-mL flasks in a Labline orbital shaker (Melrose Park, IL) at 32°C and 180 rpm. The seed culture was in late logarithmic growth after about 2 d, at which time its optical density at 440 nm was normally 9 to 10. One milliliter of 48-h seed culture was inoculated into 100 mL of medium containing the substrate lipid to be tested. The test cultures were grown for 7 d under the same conditions used for the seed culture.

Residual oleic or linoleic acid was separated from the culture, and the yeast oil was extracted according to Hammond *et al.* (12) by sequential extraction with ethanol, hexane and benzene. Fatty acid mixtures having palmitic and stearic acid were emulsified with 5 g/L gum acacia by blending after sterilization, and agitation was decreased to 140 rpm. When emulsified substrates were used, the yeast cell mass could not be separated completely; so after removal of as much of the cell mass as possible by centrifugation, the supernatant was evaporated in a rotary evaporator, and the residue was pooled with the cell mass recovered by centrifugation. Extraction of lipid in the pooled cel mass was accomplished as before.

The amount of triglyceride in yeast oil was determined by thin-layer chromatography (13). Aliquots of the ethanol extract and pooled hexane and benzene extracts were applied to layers 1.0 mm thick. The plates were developed in hexane/diethyl ether/acetic acid (50:50:1, by vol), and bands were visualized by spraying with 0.2% dichlorofluorescein in ethanol and viewed under ultraviolet light. The triglyceride bands were scraped from the plates and eluted with diethyl ether, and the residue was weighed after evaporation of the ether under nitrogen.

Stereospecific analysis was done according to Christie and Moore (14). For fatty acid analysis, glycerides were transesterified by the method of Frey and Hammond (15), and the methyl esters were analyzed on a Varian Model 3700 Gas Chromatograph (Sugarland, TX) equipped with a 1.8 m × 3.3 mm column of 10% SP-2330 on Chromosorb WAW (Supelco, Bellefonte, PA) and a flame-ionization detector.

RESULTS AND DISCUSSION

Structure of triglycerides from A. curvatum *grown on oleic acid–linoleic acid mixtures.* Table 1 shows the fatty acid composition and stereospecific distribution of the acyl groups in triglycerides from *A. curvatum* grown on pure oleic acid or linoleic acid and on mixtures of oleic and linoleic acids as carbon sources. Saturated acyl groups in the yeast triglycerides amounted to <6% in all instances. Figure 1 shows the percentages of oleate and linoleate in the yeast triglycerides plotted *vs.* the percentages of these fatty acids in the substrate. The intercept of the oleate line is very near zero, but that of the linoleate line is about +8%. Seemingly, linoleate is accumulated in slightly greater amounts than oleate from binary mixtures of the acids, and when one of the pure acids was the substrate, there was greater conversion of oleate to linoleate than of linoleate to oleate by the yeast. The plots had almost the same slopes and correlation coefficients (0.892 and 0.9988 for oleate and 0.900 and 0.9984 for linoleate, respectively).

TABLE 1

Stereospecific Analysis of Triglycerides from *Apiotrichum curvatum* Grown on Oleic Acid and Linoleic Acid at Various Ratios

18:1/18:2 ratio		Acyl composition			
		16:0	18:0	18:1	18:2
100:0	TG[a]	0.8	0.7	92.2	6.3
	sn-1	2.4	0.6	91.1	5.9
	sn-2	—[b]	—	93.5	6.0
	sn-3	—	1.5	91.6	7.0
94:6	TG	3.4	2.6	83.5	10.5
	sn-1	N.A.[c]			
	sn-2	—	—	92.9	7.1
	sn-3	N.A.			
85:15	TG	1.3	1.0	76.9	20.8
	sn-1	3.4	1.4	66.0	29.3
	sn-2	0.5	0.2	86.6	12.6
	sn-3	—	1.4	78.1	20.5
76:24	TG	1.4	1.1	65.7	31.9
	sn-1	2.9	1.4	56.9	38.9
	sn-2	—	—	80.8	19.2
	sn-3	1.3	1.9	59.4	37.6
67:33	TG	1.1	1.3	57.1	40.5
	sn-1	2.0	1.2	50.3	46.6
	sn-2	—	—	73.0	27.0
	sn-3	1.3	2.7	48.0	47.9
45:55	TG	0.8	0.8	39.6	58.8
	sn-1	1.5	0.9	30.9	66.7
	sn-2	0.2	0.1	56.1	43.5
	sn-3	0.7	1.4	31.8	66.2
33:67	TG	0.8	1.0	29.0	69.2
	sn-1	1.6	0.9	22.8	74.8
	sn-2	—	—	43.8	56.2
	sn-3	0.8	2.1	20.4	76.6
15:85	TG	1.3	1.3	13.8	83.6
	sn-1	3.6	2.1	8.7	85.7
	sn-2	0.5	0.3	27.6	71.6
	sn-3	−0.2	1.5	5.1	93.5
10:90	TG	1.2	1.6	9.0	88.2
	sn-1	3.6	2.4	7.5	86.5
	sn-2	—	—	14.7	85.3
	sn-3	—	2.4	4.8	92.8
0:100	TG	0.9	1.5	1.3	96.3
	sn-1	2.3	1.5	0.7	95.5
	sn-2	—	—	2.2	97.8
	sn-3	0.4	3.0	1.0	95.6

[a]Triglyceride. [b]Not detected. [c]Data not available.

In the triglycerides from the yeast grown on oleic acid–linoleic acid mixtures, oleate was favored at the *sn*-2 position compared with linoleate. The same trend was reported in oat oil (8), but in many seed oils, linoleate is favored in the *sn*-2 position (3). The percentages of oleate and linoleate in the *sn*-1, *sn*-2 and *sn*-3 positions were linearly related to the total percentages of these acyl groups in the triglyceride over a certain range. For oleate, the range was approximately 15–70%, and for linoleate it was approximately 25–80%. The slopes, intercepts and correlation coefficients for the linear ranges of oleate and linoleate are given in Table 2. The sign of the intercepts (positive or negative) in Table 2 indicates whether the placement of an acyl group in a certain position is favored or not. Slopes greater than 1 indicate that, as the amount of an acyl group in the whole oil increases, there is a tendency

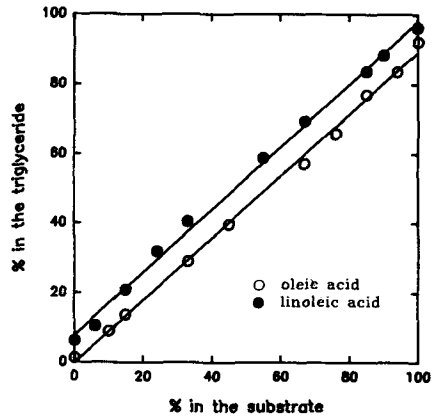

FIG. 1. The percentage of oleate and linoleate in the *Apiotrichum curvatum* triglycerides *vs.* their percentage in the substrates on which the yeast was grown. Standard linear regression lines are shown.

to place more of it in a particular glycerol position than is present in the whole oil. A slope less than 1 indicates the opposite. The slopes in Table 2 are close to 1, but slopes that deviate significantly from 1 are not unusual in vegetable oils (4,8).

Theoretical treatment of glyceride distribution. Fatemi and Hammond (4) observed that, if the slopes and intercepts of plots, such as those described in the preceding paragraph, were determined for soybean oil, the slopes for a particular acyl group for the three positions would total 3 and the intercepts would total 0. The use of plant varieties for such plots limits the range of the data to the range of fatty acid compositions that are available. Pan and Hammond (8) pointed out that, although these plots were linear over the range that could be observed, it was impossible for them to be linear over their entire range and that the lines must bend toward 0,0 and 1,1 at their extremes. The data obtained with *A. curvatum* have made it possible to examine a much longer range and to verify this prediction.

Two models are proposed to account for the data shown in Tables 1 and 2 and Figure 1. Model 1 can be used to explain the apparently linear relations, with slopes of the lines in Table 2 adding to 3.0 and intercepts adding to 0.0. The derivation is based on the simple hypothesis (Model 1) that, for some fraction of the glycerol molecules, there are enzymes that determine which acyl groups appear at the *sn*-1, *sn*-2 and *sn*-3 positions independently of the substrate concentration. This might be accomplished either by controlling which acyl group gets attached or by possible conversion of acyl groups from one form to another after attachment to the glycerol backbone. An alternate, simple model (Model 2) derived from basic chemical kinetics assumes that the glycerol positions are filled in proportion to substrate concentration but that the three *sn* positions of glycerol have different affinities for the various acyl groups. Model 2 leads to nonlinear relations, all of which pass through 0,0 and 1,1; however, Model 2 fits the seemingly linear data rather well. Both models assume that there are just two acyl groups, A and B, and that they are present in the substrate in the proportions α:β, respectively, with $\alpha + \beta = 1$. However, as a first approximation, the models can be used when more than two acyl groups are being studied, by letting A denote one specific acyl group and B denote all of the remaining acyl groups collectively. For the data in Table 1, A is identified with oleate and B with linoleate. B is favored at positions *sn*-1 and *sn*-3, and A is favored at position *sn*-2. The models can be easily adapted to other positional preferences.

TABLE 2

Linear Regression of the Percentage of an Acyl Group at the *sn* Positions of Glycerol *vs.* the Percentage of the Acyl Group in the Total Triglyceride[a]

Fatty acid	Position	Intercept	Slope	r^b
Oleate	*sn*-1	−4.76	0.94	0.9986
	sn-2	14.05	1.03	0.9992
	sn-3	−9.30	1.03	0.9991
	Sum	−0.01	3.00	
Linoleate	*sn*-1	10.02	0.93	0.9966
	sn-2	−14.04	1.01	0.9984
	sn-3	4.02	1.06	0.9994
	Sum	0.00	3.00	

[a]Ranges used for this linear regression were 13.8–65.7% for oleate and 31.9–83.6% for linoleate in the total triglyceride.
[b]Correlation coefficient.

Model 1. Assume that there are enzymes, E_1, E_2 and E_3, that control the character of the acyl groups attached to, respectively, the *sn*-1, *sn*-2 and *sn*-3 positions on certain fractions of the glycerol molecules. Assume that E_1 affects a proportion, p_1, of the glycerol molecules and ensures that acyl group B is attached to position *sn*-1. The action could be accomplished by selecting B and attaching it to *sn*-1, by blocking the attachment of A to *sn*-1, or by converting to B all acyl groups A at *sn*-1 in the fraction affected by E_1. The fraction, $1 - p_1$, of the glycerol molecules that is not affected by E_1 has A and B attached to *sn*-1 in the substrate proportions α:β. Enzyme E_2 acts at position *sn*-2 to ensure acyl group A is attached to *sn*-2 on a fraction p_2 of the glycerol molecules, and enzyme E_3 ensures that B appears at position *sn*-3 on a fraction p_3 of the glycerol molecules. It is not necessary to assume that the enzymes act independently, so that, for example, the fraction of the glycerol acted on by both E_1 and E_3 may be different from the fraction $p_1 \cdot p_3$ that would be expected from independence.

Analysis of Model 1. At position *sn*-1, the fraction p_1 of the glyceride molecules has acyl group B, and $1 - p_1$ of the molecules has acyl groups A and B distributed according to the ratio α:β. Therefore, the fraction, A_1, of glyceride molecules with acyl group A at *sn*-1 is $(1 - p_1)\alpha$. At position *sn*-2, the fraction p_2 of the molecules has A, and $1 - p_2$ of the molecules has A and B in the proportions α:β, so that the fraction A_2 of glyceride molecules with acyl group A at *sn*-2 is $p_2 + (1 - p_2)\alpha$. Analysis of position *sn*-3 is similar to *sn*-1, and the fraction, A_3, of glyceride molecules with acyl group A at *sn*-3 is $(1 - p_3)\alpha$. The fraction, A_*, of the total positions on the glyceride molecules on which fatty acid A appears is $(A_1 + A_2 + A_3)/3$. This yields Equations 1 and 2.

$$A_1 = (1 - p_1)\,\alpha$$

$$A_2 = (1 - p_2)\,\alpha + p_2$$

$$A_3 = (1 - p_3)\,\alpha \qquad [1]$$

$$A_* = \left(1 - \frac{p_1 + p_2 + p_3}{3}\right)\alpha + \frac{p_2}{3} \quad [2]$$

Because $A_* + B_* = 1$, one obtains from Equation 2:

$$B_* = \left(1 - \frac{p_1 + p_2 + p_3}{3}\right)\beta + \frac{p_1}{3} + \frac{p_3}{3} \quad [3]$$

and in a similar way one can obtain equations for B_1, B_2 and B_3 from Equations 1. Equations 2 and 3 describe linear relations as illustrated in Figure 1, in which the fraction of an acyl group in the triglyceride appears to be linearly related to the fraction of the acyl group in the substrate. Note that the slopes are equal, consistent with the observed slopes shown in Figure 1 of 0.90 and 0.892. Both intercepts are positive in Equations 2 and 3 at zero substrate concentrations, due to the hypothesized enzymatic specificity for the fatty acids. Only one intercept is positive in Figure 1, but it is this positive intercept that motivates a model in which an acyl group of a certain type is placed on the glyceride molecule independently of the amount in the substrate. The linear Equations 1 can be fit by the method of least squares to oleate data in Table 1 to get estimates of p_1, p_2 and p_3 for use in Equation 2. In a similar way, linoleate data from Table 2 can be used to get estimates of p_1, p_2 and p_3 for use in Equation 3. With these values, the graphs of Equations 2 and 3 are almost indistinguishable from the regression lines shown in Figure 1. The two sets of estimates are similar, (0.1994,0.0972,0.1672) and (0.1490,0.1001,0.1350), with the two estimates for p_2 being very close, since nearly all the acyl groups at *sn*-2 are either oleate or linoleate.

To get equations descriptive of the linear relations found in Table 2, one eliminates α between the expressions for A_i, i = 1, 2 and 3, in Equations 1 and A_* in Equation 2, and obtains Equations 4.

$$A_1 = \frac{3(1 - p_1)}{3 - p_1 - p_2 - p_3}A_* - \frac{(1 - p_1)p_2}{3 - p_1 - p_2 - p_3}$$

$$A_2 = \frac{3(1 - p_2)}{3 - p_1 - p_2 - p_3}A_* + \frac{(2 - p_1 - p_3)p_2}{3 - p_1 - p_2 - p_3}$$

$$A_3 = \frac{3(1 - p_3)}{3 - p_1 - p_2 - p_3}A_* - \frac{(1 - p_3)p_2}{3 - p_1 - p_2 - p_3} \quad [4]$$

Observe that the sum of the slopes is 3 and that the intercepts sum to 0, as is true of the data shown in Table 2. Equations 4 yield values of A_i between 0 and 1 when $p_2/3 < A_* < 1 - p_1/3 - p_3/3$. Figure 2 is a plot of the oleate on the three *sn* positions of glycerol *vs.* the oleate in the total triglyceride with the linear Equations 4 fit to the data. The lines correspond to, and are almost exactly the same as, those in Table 2. Because the equations are not linear in p_1, p_2 and p_3, nonlinear least squares fitting procedures are required.

Model 2. Assume that the affinities for A and B at the *sn*-1 position are, respectively, K(1,A) and K(1,B). The number of A acyl groups that attach to *sn*-1 will depend on the substrate fraction α and the ratio of K(1,A) to K(1,B). The fraction of A on *sn*-1 is assumed to be Equation 5.

$$\frac{K(1,A)\alpha}{K(1,A)\alpha + K(1,B)\beta} = \frac{K(1,A)\alpha}{K(1,A)\alpha + K(1,B)(1 - \alpha)} = \frac{r_1\alpha}{r_1\alpha + 1 - \alpha} \quad [5]$$

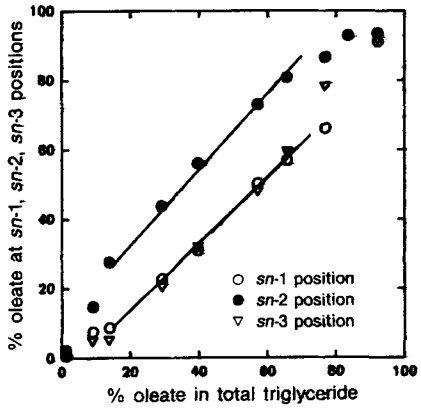

FIG. 2. The percentage of oleate in the *sn*-1, *sn*-2 and *sn*-3 positions of glycerol *vs.* the percentage of oleate in the total triglyceride fitted with Equations 4 with $p_1 = 0.2673$, $p_2 = 0.1657$, $p_3 = 0.2715$. The range of 13.8–65.7% oleate in the total triglyceride was used, as in Table 2.

where

$$r_1 = \frac{K(1,A)}{K(1,B)} \quad [6]$$

Assuming that B is preferred at *sn*-1 (meaning that the binding affinity for B at *sn*-1 is higher than for A), one would have $r_1 < 1.0$. Similar notation is assumed for *sn*-2 and *sn*-3, with $r_2 > 1.0$ and $r_3 < 1.0$.

Analysis of Model 2. From the definitions in Model 2 and of A_*, A_1, A_2 and A_3, one immediately obtains Equations 7–9.

$$A_1 = \frac{r_1\alpha}{r_1\alpha + 1 - \alpha}$$

$$A_2 = \frac{r_2\alpha}{r_2\alpha + 1 - \alpha}$$

$$A_3 = \frac{r_3\alpha}{r_3\alpha + 1 - \alpha} \quad [7]$$

$$A_* = \frac{1}{3}\left[\frac{r_1\alpha}{r_1\alpha + 1 - \alpha} + \frac{r_2\alpha}{r_2\alpha + 1 - \alpha} + \frac{r_3\alpha}{r_3\alpha + 1 - \alpha}\right] \quad [8]$$

As in Model 1, From $A_* + B_* = 1$, one obtains an expression:

$$B_* = \frac{1}{3}\left[\frac{\beta}{r_1(1 - \beta) + \beta} + \frac{\beta}{r_2(1 - \beta) + \beta} + \frac{\beta}{r_3(1 - \beta) + \beta}\right] \quad [9]$$

and in a similar way one can get equations for B_1, B_2 and B_3. In Equation 8, the relation between A_* and α is not linear, and the graph passes through 0,0 and 1,1. One can use a nonlinear minimization computer code to do a least squares fit of Equations 7 to the oleate data of Table 1 in order to get estimates for r_1, r_2 and r_3 for use in Equation 8, and a fit of a similar set of equations to the linoleate data and for use in Equation 9. Doing so, we obtain a figure analogous to Figure 1, shown in Figure 3. It is apparent from the graphs that the relations are not linear and that the intercepts are all at 0,0 and at 1,1.

One can eliminate α between Equations 7 and Equation 8 and obtain the following equations analogous to

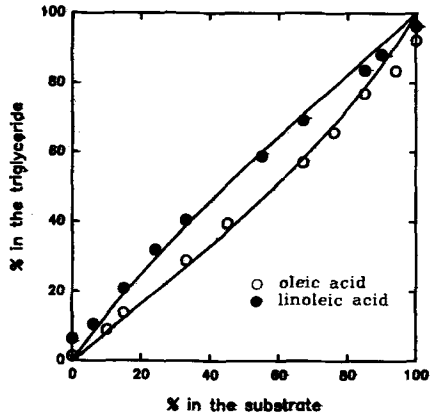

FIG. 3. The percentage of oleate and linoleate in yeast triglycerides *vs.* their percentage in the substrate with the graphs of Equations 8 and 9. The values of $r_{1,A} = 0.4651$, $r_{2,A} = 1.5460$, $r_{3,A} = 0.5041$ were obtained from fitting Equations 7 to oleate data in Table 1 and used in Equation 8. A similar procedure was used to obtain $r_{r,B} = 0.6468$, $r_{2,B} = 1.5689$, $r_{3,B} = 0.5768$ for use in Equation 9.

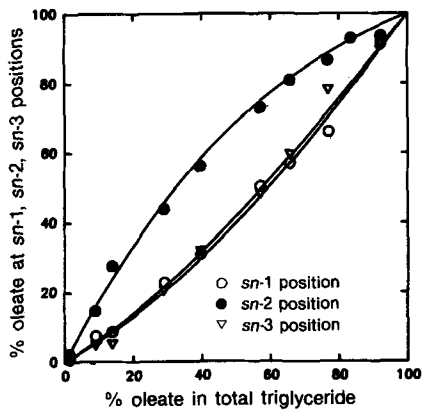

FIG. 4. The percentage of oleate in the *sn*-1, *sn*-2 and *sn*-3 positions of glycerol *vs.* the percentage of oleate in the total triglyceride fitted with Equations 10; $r_1 = 0.3146$, $r_2 = 1.0$ and $r_3 = 0.3380$.

Equations 4 and descriptive of the relations shown in Figure 2 (see Equations 10).

$$3A_* = A_1 + \frac{A_1 r_2}{(r_2 - r_1)A_1 + r_1} + \frac{A_1 r_3}{(r_3 - r_1)A_1 + r_1}$$

$$3A_* = \frac{A_2 r_1}{(r_1 - r_2)A_2 + r_2} + A_2 + \frac{A_2 r_3}{(r_3 - r_2)A_2 + r_2}$$

$$3A_* = \frac{A_3 r_1}{(r_1 - r_3)A_3 + r_3} + \frac{A_3 r_2}{(r_2 - r_3)A_3 + r_3} + A_3 \qquad [10]$$

To write A_i in terms of A_* requires computing the root of a cubic, and it is better to use a root-solving routine to compute the value of A_i, given A_*, as needed. These equations are homogeneous in the values r_1, r_2 and r_3; the relations defined for one set of values r_1, r_2 and r_3 will be exactly the same for another set of values Cr_1, Cr_2 and Cr_3 for any nonzero number C. Therefore, in fitting these equations to data, such as that represented in Figure 2, only the relative values of r_1, r_2 and r_3 will be obtained. We normalized the three equations by dividing by r_2, (equivalent to restricting r_2 to be 1.0), fit the equations to the data in the oleate column of Table 1 and obtained $r_1 = 0.3146$ and $r_3 = 0.3308$. These compare with the values in Figure 3, $r_{1,A}/r_{2,A} = 0.3008$ and $r_{3,A}/r_{2,A} = 0.3261$. The resulting curves are shown in Figure 4, where it can be seen that these curved lines fit the data very well.

In plots analogous to Figure 2, the slopes add to 3, and the intercepts add to 0. The data for Figure 2 are based on N measurements $\{A_{i,j}\}_{j=1,N}$ of oleate percentages at each position, *sn*-i, i = 1, 2 and 3, and computation of $A_{*,j} = (A_{1,j} + A_{2,j} + A_{3,j})/3$, j = 1, N. Similar plots are shown in (4,8,10,11) in which straight lines $A_i = m_i A_* + b_i$ are fit by the method of least squares to the data $\{A_{i,j}, A_{*,j}\}_{j=1,N}$ for i = 1, 2 and 3. We will show that for all such regressions, there is an algebraic identity that $m_1 + m_2 + m_3 = 3$ and $b_1 + b_2 + b_3 = 0$. From the method of least squares, the "normal equations" (16) used to obtain m_i and b_i (for i = 1, 2 or 3) are represented by the matrix equation, Equation 11.

$$\begin{bmatrix} \sum_{j=1}^{N} A_{*,j}^2 & \sum_{j=1}^{N} A_{*,j} \\ \sum_{j=1}^{N} A_{*,j} & \sum_{j=1}^{N} 1 \end{bmatrix} \begin{bmatrix} m_i \\ b_i \end{bmatrix} = \begin{bmatrix} \sum_{j=1}^{N} A_{*,j} A_{i,j} \\ \sum_{j=1}^{N} A_{i,j} \end{bmatrix} \qquad [11]$$

The matrix on the left is indepdent of i and is invertible. Adding the three equations for i = 1, 2 and 3, one obtains Equation 12.

$$\begin{bmatrix} \sum_{j=1}^{N} A_{*,j}^2 & \sum_{j=1}^{N} A_{*,j} \\ \sum_{j=1}^{N} A_{*,j} & \sum_{j=1}^{N} 1 \end{bmatrix} \begin{bmatrix} m_1 + m_2 + m_3 \\ b_1 + b_2 + b_3 \end{bmatrix} = \begin{bmatrix} \sum_{j=1}^{N} A_{*,j}(A_{1,j} + A_{2,j} + A_{3,j}) \\ \sum_{j=1}^{N} (A_{1,j} + A_{2,j} + A_{3,j}) \end{bmatrix} \qquad [12]$$

Using the definition of $A_{*,j} = (A_{1,j} + A_{2,j} + A_{3,j})/3$, one obtains Equation 13.

$$\begin{bmatrix} \sum_{j=1}^{N} A_{*,j}^2 & \sum_{j=1}^{N} A_{*,j} \\ \sum_{j=1}^{N} A_{*,j} & \sum_{j=1}^{N} 1 \end{bmatrix} \begin{bmatrix} m_1 + m_2 + m_3 \\ b_1 + b_2 + b_3 \end{bmatrix} = \begin{bmatrix} 3\sum_{j=1}^{N} A_{*,j}^2 \\ 3\sum_{j=1}^{N} A_{*,j} \end{bmatrix} \qquad [13]$$

This last matrix equation has the solution $m_1 + m_2 + m_3 = 3$ and $b_1 + b_2 + b_3 = 0$, which is unique because the matrix on the left is invertible.

Biological significance of the models. The biased distribution of acyl groups at the *sn* positions of glycerol has mostly been attributed to biases in the specificity of the enzymes involved (6). Model 2 shows that if these biases are the only factors operating, plots such as those in Figures 2 and 4 cannot be linear; however, the curvature

of the plot may be slight enough to appear linear over a short range of variation. The yeast data reported here are the only data available with enough range to demonstrate such curvature, and their fit, illustrated in Figure 4, support this model. Model 2 is also supported by reports that the amounts of individual triglycerides in seed fats agree fairly well with the amounts predicted by stereospecific analysis and 1-random-2-random-3-random distribution Equations 4.

For a truly linear outcome of plots, such as those in Figures 2 and 4, Model 1 teaches that a fixed fraction of the triglycerides must have a particular acyl group distribution that is uninfluenced by the amounts of fatty acids available for esterification. Such a distribution might occur, for example, if a fraction of the triglycerides is always formed from a certain pool of phosphatides with rigid acyl group compositions. This sort of assumption is supported by plots (*e.g.*, Fig. 1) that show a fixed bias in the incorporation of linoleate regardless of the amount available in the medium. This assumption also is supported by the seeming linearity of plots (*e.g.*, Fig. 2) for many seed oils. This model also might account for nonrandom biases in acyl group distribution that cannot be attributed to stereospecific distributions, such as those reported for olive oil (5).

Effect of substrate mixtures containing saturated fatty acid on the composition of yeast triglycerides. The other binary mixtures of fatty acids could not be studied over the wide range used for oleic acid–linoleic acid mixtures because the acyl group composition of *A. curvatum* triglycerides varied over a relatively narrow range, regardless of the ratios of fatty acids in the substrate. Also the emulsions of solid, saturated, high-melting acids used as substrates made it difficult to isolate the yeast triglyceride for stereospecific analysis. Table 3 shows the fatty acid composition of triglycerides isolated from *A. curvatum* grown on various binary combinations that included saturated fatty acids as carbon sources. When large proportions of stearic acid were present in the medium, the amount of accumulated yeast oil decreased (data not shown) in agreement with the previous observations by Lee *et al.* (13). Less than 10% stearic acid was observed in the yeast triglycerides even from a substrate with 75% stearic acid. Seemingly, the yeast reluctantly used and incorporated stearic acid into its triglycerides. In a study using cell-free extracts and spheroplasts, Holdsworth and Ratledge (17) reported that the activity of fatty acyl coenzyme A (CoA) synthetase in *A. curvatum* was some 6- to 8-fold lower with stearic acid than with palmitic, oleic and linoleic acids. The apparently poor substrate activity of this enzyme with stearic acid might account for the very limited utilization of stearic acid and its low incorporation into the triglyceride. In the stearic acid–oleic acid and palmitic acid–oleic acid mixtures, the presence of saturated acids seemed to decrease by 2–4% the linoleate found in the yeast triglyceride compared with that found for pure oleic acid as a substrate.

Although Holdsworth and Ratledge (17) reported that the activities of fatty acyl CoA synthetase for palmitic and linoleic acids were similar, our microscopic observation of the yeast showed that they accumulated less lipid as the proportion of palmitic acid increased in the substrate. In palmitic acid–stearic acid mixtures, the yeast seemed to prefer palmitic over stearic acid because the yeast triglyceride contained much more palmitate than stearate. With palmitic acid–oleic acid or palmitic acid–linoleic acid substrates, the yeast contained only 15–18% palmitate when the proportion of palmitic acid was 25–50%. It is suggestive that the 15–18% palmitate found in these triglycerides corresponded to the solubility of palmitic acid in oleic acid at 33°C, namely, 15.7% (18). It appears likely that the access of the yeast to solid lipid substrate is less than for liquid fatty acids, and this influences the proportions of the acyl groups in the yeast triglyceride. The percentage of palmitate in the triglyceride increased to 25–40% when the proportion of palmitic acid in the substrate was 75%. Much more palmitate was found in combination with oleate than with linoleate.

Table 4 shows the stereospecific analyses of three triglycerides that we were able to isolate from yeast grown on mixtures containing palmitic acid and an unsaturated acid. The palmitate accumulated in the *sn*-1 and *sn*-3 positions, and the amounts of unsaturated acyl groups in these positions decreased relative to the amounts found for substrates of pure oleate or linoleate.

TABLE 3

Fatty Acid Composition of Triglyceride from *Apiotrichum curvatum* Grown on Various Combinations of Saturated and Unsaturated Fatty Acids as Carbon Sources[a]

Carbon source	Acyl composition				
	16:0	16:1	18:0	18:1	18:2
16:0/18:0 = 75:25	60.7	11.6	1.3	23.9	2.4
50:50	56.5	8.7	2.7	28.3	3.8
25:75	50.8	4.5	6.5	33.6	4.5
16:0/18:1 = 75:25	39.9	3.1	1.6	50.7	4.8
50:50	17.7	—	1.5	78.3	2.5
25:75	16.9	—	1.6	78.8	2.7
16:0/18:2 = 75:25	25.3	1.7	1.9	6.3	64.8
50:50	15.0	—	1.7	2.3	81.1
25:75	14.9	—	2.0	4.0	79.1
18:0/18:1 = 75:25	0.9	—	9.7	86.4	3.1
50:50	0.5	—	2.5	95.1	1.2
25:75	0.5	—	3.1	94.7	1.7
18:0/18:2 = 75:25	—	—	6.0	2.1	91.9
50:50	0.5	—	5.7	1.5	92.3
25:75	0.5	—	3.7	1.3	94.5
16:0 = 100	61.3	13.9	0.8	21.5	2.6
18:0 = 100	0.9	—	48.0	46.1	5.1
18:1 = 100	0.8	—	0.7	92.2	6.3
18:2 = 100	0.9	—	1.5	1.3	96.3

[a]The compositions observed for substrates of single fatty acids used in the mixtures are included for comparison.

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TABLE 4

Stereospecific Analysis of Triglycerides from *Apiotrichum curvatum* Grown on Mixtures of Palmitic Acid and Oleic Acid or Linoleic Acid

Carbon sources		Acyl composition				
		16:0	16:1	18:0	18:1	18:2
16:0/18:1 = 75:25	TG[a]	42.0	2.5	1.4	51.4	2.7
	sn-1	62.4	3.2	1.8	30.5	1.9
	sn-2	2.4	2.7	—[b]	88.7	5.4
	sn-3	61.0	1.6	2.4	35.0	0.8
16:0/18:2 = 75:25	TG	25.2	1.2	2.0	6.3	65.4
	sn-1	37.3	1.4	3.2	3.3	56.2
	sn-2	1.6	1.2	—	11.6	84.6
	sn-3	36.7	1.0	2.8	4.0	55.4
16:0/18:2 = 25:75	TG	15.1	—	2.2	4.1	78.6
	sn-1	26.6	—	2.6	2.1	68.8
	sn-2	0.8	—	—	6.0	93.4
	sn-3	17.9	—	4.0	4.2	73.6

[a]Triglyceride. [b]Not detected.

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Different Mechanisms of Uptake of Stearic Acid and Cholesterol into Rabbit Jejunal Brush Border Membrane Vesicles

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The rate of uptake of stearic acid and cholesterol solubilized in taurocholic acid (TC) was examined in rabbit jejunal brush border membrane vesicles (BBMV). For stearic acid (18:0) or cholesterol there was an initial rapid rate of uptake, which reached a plateau within approximately 1 min and remained stable thereafter. At low concentrations of 18:0 and 20 mM, but not 2 mM, TC, there was a curvilinear relationship between the concentration of 18:0 and uptake, whereas the relationship between cholesterol uptake and concentration was linear over a wide range of values. When the concentration of TC was held constant at increasing concentrations of 18:0 or cholesterol, there was a linear increase in the rate of uptake. When the concentration of 18:0 or cholesterol was held constant and the concentration of TC was increased from 2 to 20 mM, the uptake of 18:0 declined, but the rate of uptake of cholesterol increased. When the concentrations of 18:0 plus TC, or cholesterol plus TC, were both increased in unison and their ratio was held constant, their rate of uptake increased. Thus, (i) BBMV may be used to assess the rate of uptake of lipids; (ii) the partitioning of cholesterol from bile acid micelles into the BBMV appears to be by way of "collision" of the cholesterol with the membrane. In contrast, the uptake of 18:0 from the micelle into the membrane vesicles may be by both the collision and the aqueous/dissociation models; and (iii) 18:0 uptake may be mediated by both a concentration-dependent and a concentration-independent component. Thus, stearic acid and cholesterol seem to be taken up from bile acid micelles into rabbit BBMV by different mechanisms.
Lipids 28, 1063–1067 (1993).

The intestinal uptake of lipids involves a variety of mechanisms by which lipids interact with the brush border membrane (BBM) of the small intestine (1–4). Uptake of lipids is thought to occur passively *via* an energy-independent diffusion process (5,6). A membrane fatty acid binding protein may be involved in fatty acid absorption in the intestine (7), and the uptake of both cholesterol and phosphatidylcholine may also be protein-mediated in rabbit small intestine (8,9).

The mechanism by which lipid is transferred from the bile acid micelle to the BBM has been studied using *in vitro* experiments. These studies have provided evidence for the movement of lipid from the micelle into the aqueous phase, with the lipid then permeating into the BBM (10,11). Studies using intestinal BBM vesicles (BBMV) have suggested that some portion of the uptake of lipids results from collision of micelles with the BBMV (8,12,13). BBMV are a useful model to study the uptake of lipid in the absence of an unstirred water layer and of cytosolic fatty acid binding proteins.

The aim of this study was to examine the uptake of stearic acid and cholesterol into jejunal BBMV of rabbits under conditions of varying concentrations of bile acid, and varying ratios of bile acid to stearic acid or cholesterol. The results suggest that stearic acid and cholesterol may be taken up from bile acid micelles into intestinal BBMV by different mechanisms.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Stearic acid (55.3 mCi/mmol) and [^{14}C]cholesterol (55 mCi/mmol) were purchased from ICN Radiochemicals (Irvine, CA). Phloretin, *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (HEPES), Tris-HCl and taurocholic acid (TC) were obtained from Sigma Chemical Co (St. Louis, MO). All other chemicals were reagent grade.

Isolation of BBMV. BBMV were prepared at 4°C using a modification (14) of the method described by Ling *et al.* (15). Female New Zealand white rabbits weighing 2.0–2.5 kg were given an injection of sodium pentobarbital (240 mg/kg) into the marginal ear vein.

Approximately 80 cm of jejunum distal to the ligament of Treitz was removed and flushed three times with 60 mL of 0.9% ice cold saline. The tissue was then placed on a pre-chilled glass slide and moistened with a small volume of Tris-mannitol buffer (50 mM mannitol, 2 mM Tris, pH 7.1). The mesenteric border was removed and the intestine was opened, exposing the mucosal surface. The mucosa was removed by gently scraping with two glass slides and placing the scrapings into a vial containing 5 mL isolation buffer (300 mM D-mannitol, 10 mM HEPES-Tris, pH 7.0). All steps were done at 4°C. The scrapings were transferred to a 250-mL polypropylene bottle with an additional 110 mL of isolation buffer homogenized using a Polytron® (Brinkman Instruments, Westbury, NY) at setting "9" for 30 s. The homogenate was centrifuged at 500 × *g* for 15 min (Beckman J2-21, JA-14 rotor, Beckman Instruments, Fullerton, CA). Sufficient 1M $CaCl_2$ was added to the supernatant to yield a 10 mM concentration; the mixture was then stirred on ice for 10 min. Following precipitation, the solution was centrifuged at 7500 × *g* for 20 min (JA-14 rotor). The supernatant was decanted into 50-mL polypropylene tubes and centrifuged at 28000 × *g* for 20 min (JA-20 rotor). The supernatant was then discarded, and the pellet was resuspended in vesicle resuspension buffer (300 mM D-mannitol, 10 mM HEPES-Tris, pH 7.4) and homogenized at setting "8" for 30 s. The homogenate was centrifuged at 30000 × *g* for 20 min (JA-20 rotor). The supernatant was discarded and the pellet resuspended in an appropriate volume of resuspension buffer and homogenized at setting "6" for 15 s. BBMV were incubated at 4°C for 90 min before uptake studies were done. The protein concentrations were determined using Hartree's modification (16) of the Lowry method (17) using bovine serum albumin as standard.

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Abbreviations: BBM, brush border membrane; BBMV, brush border membrane vesicles; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; TC, taurocholic acid.

The purity of the BBMV was examined measuring sucrase and alkaline phosphatase activities (18,19). The final BBMV were enriched 10- to 15-fold.

Preparation of the stearic acid probe. [^{14}C]Stearic acid was combined with unlabelled stearic acid, dried down under a stream of nitrogen and reconstituted in lipid transport buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 100 mM D-mannitol, 10 mM HEPES-Tris, 20 mM TC, pH 7.4) such that the final concentration of 18:0 was 50 or 200 μM, and the final specific activities were 8.8 and 9.8 Ci/mol, respectively. The final probes were sonicated for 15 min in a water-bath sonicator (Branson 1200; Branson Ultrasonic, Danbury, CT) at 37°C.

Preparation of the cholesterol probe. [^{14}C]Cholesterol was combined with unlabelled cholesterol and dried down under a stream of nitrogen and reconstituted in lipid transport buffer, yielding a final concentration of 50 μM and a final specific activity of 20.8 Ci/mol. The probe was sonicated for 15 min in a water-bath sonicator.

Preparation of filter prewash. Stearic acid (300 μM) was solubilized in stock stop buffer (0.15 M KCl, 2 mM HEPES-Tris, pH 7.4) containing 30 mM TC. Similarly, cholesterol (300 μM) was solubilized in stock stop buffer containing 20 mM TC. All filter prewash solutions were stirred at 48°C until the solutions cleared.

Uptake studies. Transport studies were performed using a method of rapid filtration as described by Hopfer *et al.* (20). All incubations were done at room temperature (20°C). For incubation times less than 1 min, 20 μL (60 μg) vesicles and 20 μL probe were pipetted into a polystyrene tube without the two drops touching. The reaction was initiated by mixing the two drops, maintained by continuous vortexing, and stopped by adding 1.25 mL of ice cold stop buffer (0.15 mM KCl, 2 mM HEPES-Tris, pH 7.4).

For incubation times greater than 1 min, 50 μL of vesicles (150 μg) was added to a polystyrene tube containing 50 μL of labelled substrate. The mixture was vortexed and allowed to stand for the duration of the incubation. To stop the reaction, 40 μL of the incubation mixture was added to 1.25 mL of ice-cold stop buffer. One mL of the stopped mixture was filtered through prewashed 0.45 μM cellulose filters (Millipore, Bedford, MA; Micron Sep, Fisher E04WPO2500, Cincinnati, OH) under vacuum on an Amicon (Danvers, MA) filtration manifold VFMI. Similarly, blanks were prepared by adding 20 μL of probe and 20 μL of vesicle resuspension buffer followed by 1.25 mL ice cold stop buffer.

Standards were prepared by aliquoting 50 μL of incubation mixture that was pooled from the experiment onto a pre-washed filter. The filters were placed in vials and then dried at 55°C for 10 min, followed by the addition of 7 mL scintillation fluid (Beckman scintillation cocktail).

Radioactivity was determined using a Beckman LS 9000 liquid scintillation counter.

Experimental procedures. Stearic acid (25 or 100 μM) or cholesterol (25 μM) uptake was studied as a function of time. The lipids were solubilized in 20 mM TC and were incubated with BBMV suspension for 5-s to 16 min. Stearic acid was examined at concentrations of 1 to 200 μM, and cholesterol was studied at increasing concentrations of 1 to 50 μM.

Stearic acid (25 or 100 μM) and cholesterol (25 μM) were examined at 5 s incubation periods with TC concentrations ranging from 2 to 20 mM. Stearic acid uptake with TC at fixed ratios of 12.5:1 or 1.25:1 (μM 18:0 mM TC) and cholesterol (1.25:1) were examined at initial rates of 5-s incubation periods. Stearic acid (25 μM) and cholesterol (25 μM) uptake was studied when the pH of the vesicle resuspension buffer and lipid probe were maintained at pH 4.0, 6.0 and 7.4; the concentration of TC was 20 mM, and the duration of incubations was 5 s.

The data for each point on the subsequent graphs represent the mean value ± standard error of four experimental days on which four replicates were performed. Uptake in the time-course experiments was expressed as nmol/mg BBMV protein; it represents the total radioactivity of the sample minus the nonspecific binding of labelled lipid to the filter. To determine the best curve fit, Sigmaplot (4.1) graphics program (Jandel Scientific, San Rafael, CA) was used for the time-course experiments. For the concentration curves, lipid/TC ratio (constant ratio of lipid/TC with increasing concentrations of both) and constant concentration of lipid with increasing TC, the uptake is presented as nmol per mg BBMV protein per second. First-order regressions and regression coefficients were calculated for experiments done under initial rate conditions (5 s).

RESULTS

Time course of stearic acid and cholesterol. Studies of 100 μM stearic acid (18:0) uptake as a function of time showed an initial rate of uptake that plateaued within 2 min at a value of approximately 35 nmol per mg BBMV protein (Fig. 1A). Similarly, 25 μM 18:0 (Fig. 1B) and 25 μM cholesterol (Fig. 1C) showed a rapid initial rate of uptake, equilibrating within 2 min at values of 3 and 4 nmol per mg protein, respectively.

Lipid concentration curve. The uptake of 18:0 was greater from low (2 mM) than from high (20 mM) concentrations of TC (Fig. 2A and 2B). The relationship between the concentration of 18:0 and uptake was linear when the 18:0 was solubilized with 2 mM TC (Fig. 2A), but a curvilinear relationship was noted at low concentrations of 18:0 when the fatty acid was solubilized with 20 mM TC (Fig. 2B). For cholesterol, there was a linear relationship between concentration and uptake (Fig. 2C).

Lipid and TC varied to maintain constant ratio. The concentrations of 18:0 and TC were both increased in unison, maintaining a constant ratio of 12.5 μM/1 mM (Fig. 3A) or 1.25 μM/1 mM (Fig. 3B); there was a linear increase in 18:0 uptake. Similarly, cholesterol uptake increased at a linear rate as both cholesterol and TC concentrations were increased at a constant ratio of 1.25 μM/1 mM (Fig. 3C).

Constant lipid concentration with increasing TC. The uptake of 100 or 25 μM 18:0 decreased as the concentration of TC was increased from 2 to 20 mM (Fig. 4A and 4B), whereas cholesterol uptake increased as the TC concentration was increased (Fig. 4C).

DISCUSSION

Several mechanisms have been postulated to explain the sequence of events occurring when lipid is transferred from bile salt micelles in the intestinal lumen into the BBM. Uptake of the entire micelle is not supported by experimental data (21). The second model involves a "collision" between the micelle and the BBM whereby the solubilized lipid moves directly from the micelle into the BBM. In the third model, the movement of lipid occurs

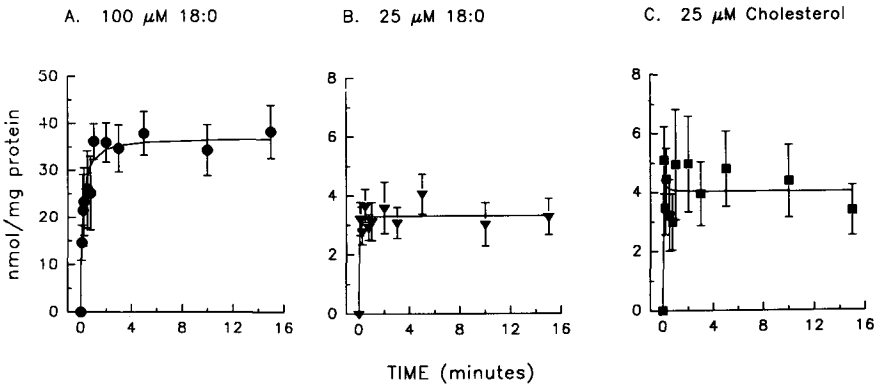

FIG. 1. Rate of uptake (mean ± SEM) of stearic acid and cholesterol into brush border membrane (BBM) vesicles. The concentration of stearic acid was 100 μM (panel A) or 25 μM (panel B), and the concentration of cholesterol was 25 μM (panel C). The lipids were solubilized in 20 mM taurocholic acid. BBM vesicles were equilibrated with vesicle resuspension buffer for 90 min prior to experimentation. BBM vesicles were incubated with labelled stearic acid or cholesterol at room temperature (20°C). Assays were performed in quadruplicate on BBM vesicles from four rabbits in each group.

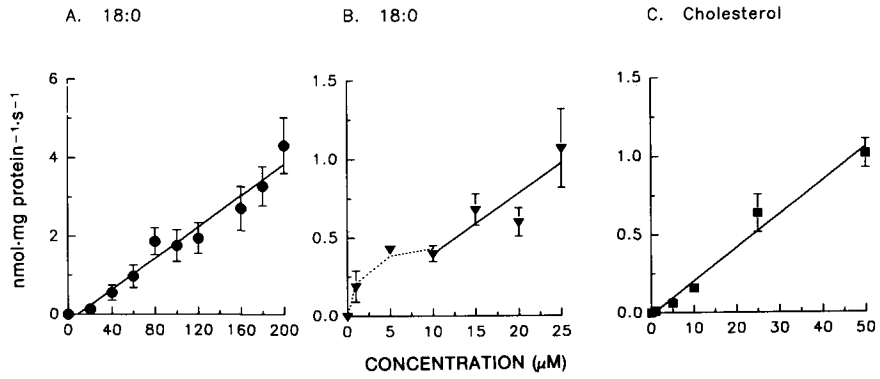

FIG. 2. Effect of varying concentration of 18:0 and cholesterol on uptake into brush border membrane (BBM) vesicles. The concentration of stearic acid (18:0) or cholesterol was varied, and the concentration of taurocholic acid was 2 mM (panel A) or 20 mM (panels B and C). The duration of incubation was 5 s. Each point represents the mean ± SEM of the results of quadruplicate measurements on BBM vesicles obtained from four rabbits in panels A and B and five rabbits in panel C.

via transfer from the micelle to the aqueous phase followed by permeation into the BBM. This "monomer" or "aqueous" hypothesis is based on an equilibrium between fatty acids that exist in monomeric form and in a micellar form (10). In solution there is a maximum concentration of monomers and a finite amount of lipid that can be solubilized into the micellar form. When the solubilizing agent has reached the total carrying capacity for the lipid, any further increase in the lipid concentration will result in the formation of an emulsion (22,23). The monomeric and micellar forms are present in the bulk phase in the intestinal lumen and in the unstirred water layer. As the monomeric form of the lipid permeates into the membrane, there is further partitioning of lipid from the micelle into the water phase.

A variation of the aqueous model, the "dissociation" model, suggests that fatty acids in a low pH microclimate just external to the BBM will be protonated, resulting in increased permeation through the lipid membrane (24). The presence of an acid microclimate has been demonstrated *in vitro* and *in vivo* (25,26). Stremmel *et al.* (7) showed that binding of oleate to rat jejunal microvillus membranes increased at pH values below 6.8 with a maximum at pH 4.0; at pH values above 8.0, there was a decrease in binding. Shiau (27) studied fatty acid uptake in everted rat jejunal sacs and demonstrated increased fatty acid uptake at lower pH values. He interpreted this data to suggest that the pH microclimate provides an environment in which fatty acid preferentially partitions from the micelle. Stearic acid is protonated in a low-pH environment and becomes more lipid soluble, whereas cholesterol is not protonated. Thus, the "dissociation" model may explain the uptake of stearic acid but not of cholesterol.

In addition to the passive permeation of lipids through the BBM, there may be a mediated component of this

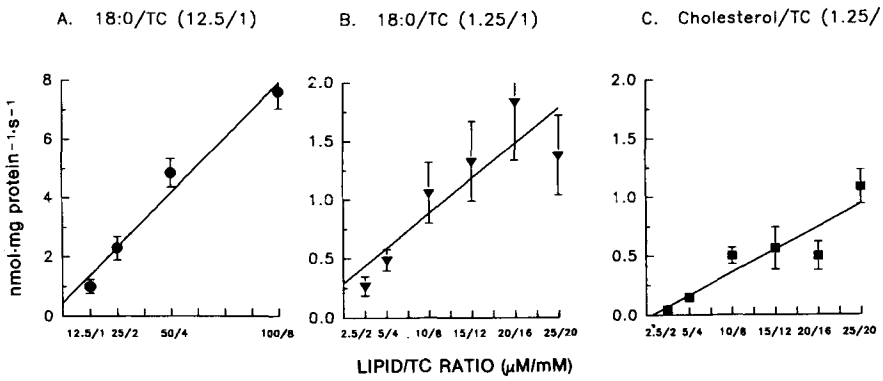

FIG. 3. Effect of increasing concentrations of both stearic acid (18:0) and taurocholic acid (TC), or cholesterol and TC, with constant ratio of stearic acid/TC or cholesterol/TC, on uptake of 18:0 or cholesterol into brush border membrane (BBM) vesicles. The duration of incubation was 5 s. The ratio of probe to TC was 12.5 μM/1 mM for 18:0 (panel A), or 1.25 μM/1 mM for 18:0 or cholesterol (panels B and C). Each point represents the mean ± SEM of the results of quadruplicate measurements on BBM vesicles obtained from four rabbits in panel A and eight rabbits in each of panels B and C.

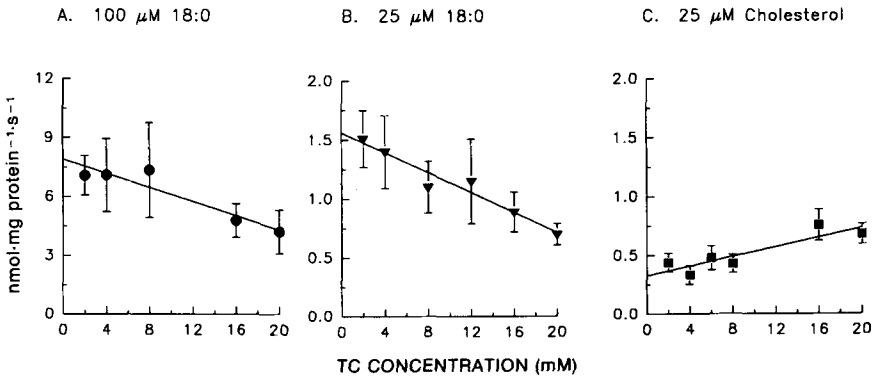

FIG. 4. Effect of varying concentrations of taurocholic acid (TC) on the uptake of 100 μM (A) or 25 μM (B) stearic acid or 25 μM cholesterol into brush border membrane (BBM) vesicles. The duration of incubation was 5 s. Each point represents the mean ± SEM of the results of quadruplicate measures on BBM vesicles obtained from five rabbits in panel A, six rabbits in panel B, and eight rabbits in panel C. The slope and correlation coefficient for panesl A, B, and C are −0.018, 0.937; −0.042, 0.972; and 0.020, 0.884, respectively.

process. Recent findings with linolenic acid uptake into rabbit jejunal BBMV demonstrated an uptake with a sodium-dependent "overshoot", suggesting that there may be a protein-mediated component involved in fatty acid uptake (15). This overshoot has not been confirmed (14), but this may be due to technical difficulties between the two studies. The use of the BBMV overcomes an effect of cytosolic fatty acid binding proteins (7) and of unstirred layers, and allows the comparison of the collision *vs.* the aqueous/dissociation models of lipid uptake. Under the conditions of low concentration of 18:0 and 20 mM TC, but not 2 mM TC, a curvilinear relationship was noted between 18:0 concentration and uptake (Fig. 2B). This suggests that under certain experimental conditions there may be a linear and a nonlinear component describing fatty acid uptake, in contrast to the single component describing cholesterol uptake under the same conditions (Fig. 2C). Caution must be used with this interpretation, however, since the regression line between 10–25 μM 18:0 intersects with the origin and since this curvilinear relationship was not obtained at equilibration (4 min or beyond).

The increasing uptake of 18:0 and cholesterol with increasing concentrations of both lipid and solubilizing TC (but with a constant ratio of lipid/TC) (Fig. 3) is suggestive of partitioning of fatty acid or cholesterol from the micelle directly into the BBMV (28), the so-called "collision" model. This same lipid/lipid model is supported by the finding of an increasing uptake of cholesterol with increasing concentrations of TC (Fig. 4C). In contrast, the reduction in the intake of 100 or 25 μM 18:0 with increasing concentrations of TC (Fig. 4A and 4B) supports, but does not prove the model of partitioning of the fatty acid from the micelle into an aqueous phase, and then into the BBM (28), the so-called aqueous/partitioning model. Again, one must be cautious with these interpretations, since the increasing cholesterol uptake with increasing concentrations of TC (Fig. 4C) may reflect increasing amounts of TC enhancing the solubility of cholesterol in the micelles; the fact that there is no derivation from the linear relation-

ship in the uptake of cholesterol above and below the critical micellar concentration of TC suggests that cholesterol solubility is present over the concentration range tested. On the other hand, the effect of increasing the amount of TC *vs.* 18:0 (Fig. 4A and 4B) might be explained by increasing competition of TC micelles for 18:0 *vs.* the BBMV. We do not have data on the activity or solubility of 18:0 in the different solutions, so that our data supports, but does not prove one or the other model. Notwithstanding, there appeared to be qualitative differences in the uptake of stearic acid and cholesterol (Figs. 3 and 4): the partitioning of cholesterol from the bile acid micelle into the BBM appears to be by way of the "collision" of the cholesterol into the membrane. In contrast, the uptake of stearic acid from the micelle into the BBM may be by both the collision and the aqueous/dissociation models, in which the acid microclimate may play an additional role. Furthermore, the curvilinear relationship between concentration and uptake of low concentrations of 18:0 solubilized in 20 mM TC, but a linear relationship for cholesterol under the same conditions further suggests that stearic acid and cholesterol may be taken up by different mechanisms from bile acid micelles into the intestinal BBM.

The uptake of D-glucose into BBMV is not affected by 2 mM TC, but is reduced by 20 mM TC (14,15). We were aware, therefore, of the possibility that the higher concentrations of TC might open the sealed vesicles and dissipate the sodium gradient. This might be one of several possible explanations for our not demonstrating an "overshoot" in the uptake of 18:0 or cholesterol (Fig. 1). However, it was at the condition of 20 mM TC that there was a curvilinear relationship between concentration and uptake of 18:0 (Fig. 2A), so that a possible leakiness of the vesicles due to TC could not explain the lack of a curvilinear concentration/uptake curve for cholesterol, or 18:0 at a lower concentration of 18:0 (Fig. 2A and 2C). Furthermore, any possible effect of TC on BBMV integrity did not produce a break in the linear relationship between the ratio of lipid/TC and uptake (Fig. 3), nor could the possibility of leakiness explain the qualitatively different relationship between the uptake of 18:0 or cholesterol when the concentration of TC is increased (Fig. 4). We recognize, of course, that the data suggesting different mechanisms for the uptake of stearic acid and cholesterol apply only to the special conditions of BBMV and may not necessarily apply to the intact intestine *in vivo*.

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Effects of *trans* Fatty Acids on Lipid Accumulation in 3T3-L1 Cells

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Previous work had shown that dietary *trans* fatty acids (*t*FA) resulted in decreased fat deposition in adipose tissue. This study was conducted to see if *t*FA influence lipid accumulation in Swiss mouse fibroblast 3T3-L1 cells, which are widely used as an adipocyte model. Cells were cultured in the presence of experimental or control growth media supplemented with fatty acids complexed to bovine serum albumin. Fatty acid compositions of experimental and control growth media were similar except that the octadecenoates in the control growth media were *cis* fatty acids, whereas those in the experimental media contained both *cis* and *trans* fatty acids. Cell-conditioned media and cellular lipids at the preadipocyte and differentiating adipocyte stages were analyzed. At both stages of development, less fat accumulated in cells cultured in the presence of *t*FA, due primarily to a decrease in the nonpolar lipid content of cells exposed to *t*FA, and linoleate to arachidonate ratios were higher in cells supplemented with *t*FA. Calculations comparing sums of saturated and monounsaturated fatty acids in cells at the differentiating adipocyte stage suggested that *t*FA may have replaced monounsaturated fatty acids in the nonpolar lipid fraction and saturated fatty acids in the polar lipid fraction. The results of these studies are in good agreement with the *in vivo* effects of *t*FA seen in previous work with mouse adipose tissue. It was concluded that the 3T3-L1 *in vitro* model is an appropriate system for further studies of *t*FA and lipid metabolism in adipose tissue.
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Partially hydrogenated fats are widely used in processed food and constitute a significant proportion of dietary lipid (1–4). These fats contain a broad range of positional isomers (5) of *trans* fatty acids (*t*FA), whose average dietary intake in the United States has been estimated to be anywhere between 7.6 to 13.3 g per capita per day (6,7).

Considerable attention has been focused on *t*FA as they are not the normal end products of lipid metabolism in animal tissues (8,9). Despite the large number of studies that have been undertaken, questions regarding the nutritional significance (10–12) and the metabolic fate and effects of *t*FA remain to be answered. It is clear, however, that some of the physiological properties of *t*FA are substantially different from those of the corresponding *cis*-unsaturated isomers. Recent dietary studies (13–16) have demonstrated that in contrast to *cis*-monounsaturated FA, *t*FA raise low density lipoprotein (LDL) cholesterol, lower high density lipoprotein (HDL) cholesterol and raise levels of plasma lipoprotein (a). *In vitro* studies (17) have provided evidence that *cis*- and *trans*- octadecenoates have opposite effects on cholesteryl ester transfer protein activity. As a consequence of these recent studies, there is renewed interest in the possible role of *t*FA in health and disease.

Most studies involving metabolism of *t*FA have employed laboratory animals. In our laboratory, we have used the C57Bl/6J mouse as a model for dietary studies of *t*FA. Work with this model has demonstrated an ability of diets containing *t*FA to affect lipid accumulation in milk fat (18) and in adipose tissue (19). In these studies, diets containing *t*FA depressed the percentage of fat in mouse milk (18) and decreased epididymal fat pad weight, perirenal fat weight, epididymal fat cell size and the triacylglycerol-to-polar-lipid ratio in epididymal fat pads (19).

The present study was conducted to see if *t*FA affect lipid accumulation in 3T3-L1 cells, a subclone of Swiss mouse embryo 3T3 fibroblasts. The 3T3-L1 cell line was selected for study because it has been reported to differentiate into cells with biochemical and morphological characteristics of adipocytes (20–23), and it has been widely studied as a model for adipocyte differentiation (24–26) and the regulation of lipid metabolism (27–33).

MATERIALS AND METHODS

Materials. The 3T3-L1 cells were generously provided by Dr. Juan Calvo (National Institute of Health, Bethesda, MD). Low protein serum replacement (LPSR-1) (serum supplement), essentially FA-free bovine serum albumin (BSA), fetal calf serum (FCS), 3-isobutyl-1-methyl xanthine (MIX) and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) was purchased from Life Technologies (Gaithersburg, MD). Trypsin-Versene mixture, L-glutamine, penicillin–streptomycin–fungizone mixture, sodium pyruvate and nonessential amino acid mixture (NEAA) were purchased from Whittaker M.A. Bioproducts (Columbia, MD). All fats (cocoa butter, corn oil, olive oil and margarine) for the experiments were obtained locally. Authentic FA methyl ester (FAME) standards were purchased from Nu-Chek-Prep (Elysian, MN).

Preparation of FA-supplemented media. DMEM supplemented with 2 mM glutamine, 0.1 mM of each NEAA, 1 mM sodium pyruvate, 100 units of potassium penicillin G/mL, 100 μg streptomycin sulfate/mL and 0.25 μg fungizone/mL, 3% FCS and 2% LPSR-1 was designated as growth medium (GM). FA-supplemented media were prepared as described by Grunfled *et al.* (34). FA were derived by saponification of an experimental fat (which was obtained by decanting a melted corn oil margarine) and a control fat (which was made from a mixture of corn oil, olive oil and cocoa butter). After saponifying the fats in 0.6 N NaOH in methanol at 37°C for 1 h and removing the nonsaponifiable material by extracting the reaction mixture with 3 × 1 vol of hexane, the FA were obtained by acidifying the FA sodium salts with 6N HCl and extracting the mixture with hexane. Hexane was removed

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Abbreviations: BSA, bovine serum albumin; cAMP, 3′,5′-cyclic adenosine monophosphate; CGM, control growth medium; DA, differentiating adipocytes; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle medium; EGM, experimental growth medium; FAME, fatty acid methyl esters; FCS, fetal calf serum; GM, growth medium; HDL, high density lipoprotein; LDL, low density lipoprotein; LPSR-1, low protein serum replacement; MIX, 3-isobutyl-1-methyl xanthine; NEAA, nonessential amino acids; PA, preadipocytes; PBS, phosphate buffered saline; *t*FA, *trans* fatty acids; TLC, thin-layer chromatography; 3T3-L1, subclone of Swiss mouse embryo 3T3 fibroblasts.

by vacuum evaporation, and the FA were dissolved in ethanol. FA (0.2M) in ethanol were diluted 1:25 with phosphate buffered saline (PBS), pH 7.4, containing 20% BSA, and heated at 55°C for 45 min. The mixture was gently agitated until the solution was clear. The pH of the FA-albumin solution was then adjusted to 7.4 with NaOH. This procedure resulted in a noncovalent complex of FA to BSA in an FA/BSA molar ratio of no greater than 3:1. The FA-albumin complex was then diluted 1:10 with GM and then filter-sterilized. GM supplemented with control or experimental FA are referred to as control growth medium (CGM) and experimental growth medium (EGM), respectively.

Cell culture. Stock cultures of 3T3-L1 cells were maintained in 75-cm^2 culture flasks in GM. Cultures were maintained in an atmosphere of 5% CO_2, 95% air at 37°C and were fed with 12 mL of medium every four days. Cell monolayers at near confluence were trypsinized (1% Trypsin-Versin at 37°C for 30 s), split 1:4, supplemented with 12 mL of either CGM or EGM, and allowed to grow for 96 h to reach confluence. Confluent cells cultured in the presence of CGM and EGM were designated as preadipocytes (PA). The day cells reached confluence in the presence of CGM and EGM was referred to as Day 0, with respect to differentiating adipocytes (DA). On Day 0, cell-conditioned media were removed, and 12 mL of CGM or EGM containing 0.5 mM MIX and 0.25 μM DEX was added to stimulate differentiation as described by Rubin *et al.* (35). After 48 h (Day 2), cell-conditioned media were removed, and the cells were allowed to grow in 12 mL of fresh CGM or EGM for an additional 3 d (Day 5). The Day 5 cells are referred to as DA.

Lipid extraction and FA analysis. Cell-conditioned media on Days 0, 2 and 5 were cleared of cells by centrifugation at 200 × *g* for 3 min and lyophilized. Media were extracted with chloroform/methanol as described below. Cell monolayers were washed three times with cold PBS, pH 7.4, and harvested by centrifugation, after using a rubber policeman to help detach them from the culture flask. Each cell pellet was resuspended in 1 mL of PBS and extracted with chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene (36). Prior to analysis the total cellular lipid was separated into a nonpolar and polar lipid fraction by thin-layer chromatography (TLC) on Silica Gel G plates using diethyl ether as the mobile phase. After adding triheptadecanoin as an internal standard, the FA and fatty acyl groups in the media and cellular polar and nonpolar lipids were converted to FAME and analyzed by gas-liquid chromatography, using a glass capillary column coated with SP-2340 in a Hewlett-Packard (Avondale, PA) model 5840 chromatograph essentially as described previously (37). This involved transesterification with methanolic HCl, purification of the FAME on silicic acid TLC plates, identification of FAME by comparison of retention times with those of known standards and quantification of FAME, using appropriate response and correction factors as described elsewhere (37). The *t*-test was used to analyze the results using the Statworks (Macintosh) programs.

RESULTS

In preliminary work, cells grown in the presence of CGM and EGM were analyzed for protein by the method of Lowry *et al.* (38) and counted in a hemocytometer. Regardless of whether cells were grown in the presence of CGM or EGM, they contained similar amounts of protein and yielded similar cell counts. The protein values ranged from 2250 to 2575 μg/flask, and cell numbers were between 12 × 10^5 and 14 × 10^5 per flask.

The FA contents and compositions, as FAME, of 12 mL each of the CGM and EGM are given in Table 1. In general, the values in experimental and control media were similar, except that all of the octadecenoate fraction was *cis* in the CGM whereas about 40% of this fraction was *t*FA in the EGM. Compared to the CGM, the EGM contained significantly ($P < 0.05$) more 18:0 and slightly more total FA per mL of growth medium.

The content and composition of FA, as FAME, in polar lipids of control and experimental cells at the PA and the DA stages are summarized in Table 2. Similar data for nonpolar lipid are shown in Table 3. The *trans*-18:1 isomers (18:1*t*) were readily incorporated from the EGM into the nonpolar and polar lipid fractions isolated from the experimental cells at both the PA and DA stages of development. Small amounts of *trans* 16:1 (16:1*t*) were also observed in the cells exposed to the EGM, providing evidence that these cells were capable of at least partial oxidation of 18:1*t*. The total content of *t*FA, as well as that of other FA, was higher in the DA compared to the PA.

Compared to control cells, there was less arachidonic acid (20:4n-6) in experimental cells on either an absolute or relative basis. This was observed in both polar (Table 2) and nonpolar lipids (Table 3) and at both stages of development. Ratios of 18:2n-6/20:4n-6 were calculated, and are included in Tables 2 and 3. The ratios were significantly higher ($P < 0.05$) in experimental cells in both lipid classes for both PA and DA.

Although the EGM contained more 18:0 than CGM (Table 1), cells grown in the presence of EGM did not contain more 18:0 than the cells grown in the presence of CGM. In fact, in experimental cells, less 18:0 was observed in polar lipids of DA (Table 2) as well as in nonpolar lipids of both PA and DA (Table 3). To a certain extent, the lower amount of 18:0 in the experimental cells appeared to be compensated for by the presence of *t*FA. The extent to which *t*FA may have replaced 18:0 was evaluated by comparing the sums of saturated and monounsaturated FA

TABLE 1

Fatty Acid Content and Composition of Growth Media[a]

Fatty acid	Control		Experimental	
	μg	wt%	μg	wt%
16:0	335 ± 6	(13.7)	349 ± 5	(12.2)
16:1*c*	14.6 ± 0.3	(0.6)	10 ± 0.2	(0.4)
18:0	148 ± 3	(6.0)	229 ± 4[b]	(8.0)
18:1*c*	1133 ± 15	(46)	793 ± 16[b]	(27.8)
18:1*t*	ND		531 ± 9[b]	(18.6)
18:2n-6	767 ± 10	(31)	893 ± 13	(31)
Others[c]	48 ± 2	(2)	37 ± 1	(1.6)
Total	2452 ± 36		2852 ± 35	

[a]Values are the means ± SEM of fatty acids in μg and wt% from 12 mL of medium (n = 3); ND, not detected; *c*, *cis*; *t*, *trans*.
[b]Denotes statistically significant differences between control and experimental media at $P < 0.05$.
[c]Includes fatty acids below 0.5%, such as 14:0 and 20:4n-6.

TABLE 2

Fatty Acid Content and Composition of Polar Lipid of 3T3-L1[a]

	Preadipocytes				Differentiating adipocytes			
	Control		Experimental		Control		Experimental	
Fatty acid	μg	wt%	μg	wt%	μg	wt%	μg	wt%
16:0	19.4 ± 0.6	(19.0)	19 ± 0.3	(18.0)	24 ± 2	(17.4)	22 ± 1	(16.4)
16:1*c*	1.0 ± 0.03	(0.9)	1.9 ± 0.2	(1.8)	[b]		[b]	
16:1*t*	ND		2.5 ± 0.1[c]	(2.4)	ND		2.7 ± 0.1	(2.0)
18:0	19.4 ± 0.8	(19.0)	19.7 ± 0.4	(18.8)	36 ± 2	(26.0)	24 ± 1[c]	(18.0)
18:1*c*	21 ± 0.8	(20)	17.2 ± 1[c]	(16.4)	23 ± 2	(16.7)	24 ± 1	(18.0)
18:1*t*	ND		6.3 ± 0.7[c]	(6.0)	ND		10 ± 0.4[c]	(7.5)
18:2n-6	19.3 ± 1	(19.0)	19.7 ± 0.8	(18.8)	28 ± 2	(20.3)	32 ± 1	(23.8)
20:2n-6	0.9 ± 0.03	(0.9)	0.8 ± 0.1	(0.8)	[b]		[b]	
20:3n-6	1.1 ± 0.04	(1.1)	1.0 ± 0.1	(0.9)	1.5 ± 0.2	(1.1)	1.5 ± 0.1	(1.1)
20:4n-6	12.1 ± 0.5	(12.0)	10.6 ± 0.2	(10.1)	16 ± 2	(11.6)	13 ± 0.6[c]	(9.7)
22:0	0.7 ± 0.03	(0.7)	[b]		1.5 ±0.2	(1.1)	0.8 ± 0.1	(0.6)
22:5n-3	0.6 ± 0.02	(0.6)	0.7 ± 0.1	(0.7)	[b]		[b]	
22:6n-3	0.9 ± 0.04	(0.9)	0.8 ± 0.2	(0.9)	[b]		[b]	
24:0	1.9 ± 0.2	(1.9)	1.5 ± 0.1	(1.4)	[b]		[b]	
24:1	1.1 ± 0.04	(1.1)	0.8 ± 0.04	(0.8)	[b]		[b]	
Others[d]	2.1 ± 0.1	(2.1)	7.5 ± 0.2	(5.4)	2.1 ± 0.1	(2.1)	2.9 ± 0.2	(2.8)
18:2n-6/20:4n-6	1.6 ± 0.1		1.9 ± 0.1[c]		1.7 ± 0.1		2.5 ± 0.1[c]	

[a]Values are the means ± SEM for μg of fatty acid/culture flask and wt% for three independent determinations; ND, not detected; *c, cis; t, trans.*

[b]Values for fatty acids present at <0.5% of the total are included with Others.

[c]Denotes statistically significant differences between control and experimental cells at $P < 0.05$.

[d]Sum of fatty acids present at <0.5% ot total; includes 10:0, 12:0, 14:0, 15:0 and 17:0, as well as those indicated by *b*, above.

TABLE 3

Fatty Acid Content and Composition of Nonpolar Lipid of 3T3-L1[a]

	Preadipocytes				Differentiating adipocytes			
	Control		Experimental		Control		Experimental	
Fatty acid	μg	wt%	μg	wt%	μg	wt%	μg	wt%
12:0	0.8 ± 0.2	(1.0)	0.3 ± 0.02	(0.6)	[b]		[b]	
14:0	0.9 ± 0.2	(1.2)	0.8 ± 0.02	(1.6)	1.0 ± 0.04	(0.7)	0.7 ± 0.07	(0.9)
16:0	14 ± 0.7	(18.4)	8.4 ± 1[c]	(16.8)	22 ± 1	(16.5)	11 ± 1	(14.1)
16:1*c*	2.1 ± 0.1	(2.8)	1.1 ± 0.2	(2.2)	2.1 ± 0.2	(1.6)	2.1 ± 0.3	(2.7)
16:1*t*	ND		0.4 ± 0.1[c]	(0.8)	1.5 ± 0.1	(1.1)	3.3 ± 0.3	(4.2)
18:0	8.4 ± 0.2	(11.0)	5.5 ± 0.4[c]	(11.0)	15.2 ± 1.0	(11.4)	9.4 ± 0.3[c]	(12)
18:1*c*	24 ± 0.5	(31.6)	9.0 ± 0.5[c]	(18.0)	38 ± 3.0	(28.6)	14 ± 2[c]	(18.0)
18:1*t*	ND		5.3 ± 0.4[c]	(10.6)	ND		10.5 ± 1[c]	(13.5)
18:2n-6	15 ± 1	(19.7)	10.8 ± 0.8[c]	(21.6)	38 ± 3.0	(28.6)	20 ± 3.0[c]	(25.6)
18:3n-3	[b]		0.8 ± 0.03	(1.6)	[b]		[b]	
20:2n-6	2.2 ± 0.1	(2.9)	1.4 ± 0.2	(2.8)	1.3 ± 0.1	(1.0)	0.6 ± 0.1	(0.8)
20:3n-6	0.8 ± 0.1	(1.0)	0.4 ± 0.07	(0.8)	3.7 ± 0.5	(2.8)	1.0 ± 0.1[c]	(1.3)
20:4n-6	1.9 ± 0.5	(2.5)	0.9 ± 0.1	(1.8)	3.9 ± 0.2	(2.9)	1.4 ±0.2[c]	(1.8)
22:5n-3	1.0 ± 0.2	(1.3)	0.6 ± 0.1	(1.2)	[b]		0.8 ± 0.1	(0.8)
22:6n-3	0.6 ± 0.5	(0.8)	0.5 ± 0.03	(1.0)	[b]		[b]	
24:0	1.2 ± 0.1	(1.6)	0.8 ± 0.1	(1.6)	1.1 ± 0.1	(0.8)	0.6 ± 0.04	(0.8)
Others[d]	2.5 ±0.1	(3.3)	2.6 ± 1.0	(5.2)	5.2 ± 0.1	(3.9	2.8 ± 0.4	(3.6)
18:2n-6/20:4n-6	8 ± 0.1		12 ±1.0[c]		10 ± 0.5		14 ± 3.0[c]	

[a]Values are the means ± SEM for μg of fatty acid/culture flask and wt% for three independent determinations; ND, not detected; *c, cis; t, trans.*

[b]Values for fatty acids present at <0.5% of the total are included with Others.

[c]Denotes statistically significant differences between control and experimental cells at $P < 0.05$.

[d]Sum of fatty acids present at <0.5% ot total; includes 10:0, 15:0, 17:0 and 22:0 as well as those indicated by *b*, above.

TABLE 4

Sums of Saturated and Monounsaturated Fatty Acids in Polar and Nonpolar Lipid[a]

Fatty acids	Polar				Nonpolar			
	CPA	EPA	CDA	EDA	CPA	EPA	CDA	EDA
Total saturated	42 ± 3	40 ± 5	47 ± 6	36 ± 4[b]	35 ± 3	34 ± 5	31 ± 5	29 ± 4
Total saturated + *t*FA	42 ± 3	49 ± 5	47 ± 6	46 ± 4	35 ± 3	46 ± 4[c]	31 ± 5	48 ± 6[d]
Total monounsaturated	23 ± 6	19 ± 5	18 ± 3	18 ± 3	36 ± 4	21 ± 3[e]	32 ± 6	21 ± 4[f]
Total monounsaturated + *t*FA	23 ± 6	28 ± 4	18 ± 3	28 ± 4[g]	36 ± 4	33 ± 5	32 ± 6	40 ± 2

[a]Values are means ± SEM of wt% values for fatty acids given in Tables 2 and 3; CPA, control preadipocytes; EPA, experimental preadipocytes; CDA, control differentiating adipocytes; EDA, experimental differentiating adipocytes; *t*FA, *trans* fatty acids.
[b-g]Denote statistically significant differences between control and experimental cells at $P < 0.05$ when comparisons were made between CPA and EPA and between CDA and EDA.

in control and experimental cells, alternatively including the *t*FA as either saturated or monounsaturated FA (Table 4). The weight percent data were used to calculate these values because the contents of FA in control and experimental cells were significantly different in the nonpolar lipid fractions (see Fig. 1, discussed later). In the polar lipids of DA, but not of PA, similar levels of saturated and monounsaturated FA were found in control and experimental cells when *t*FA were included as saturated FA. However, this was not seen in nonpolar lipids, in which case the differences in the sums of saturated and monounsaturated FA became significantly different ($P < 0.05$) when *t*FA were included as saturated FA.

Except for *t*FA, and possibly 18:2n-6 in polar lipids, there was a tendency for all FA to be present in lower amounts in experimental cells. Comparisons of the sums of FA found in PA and DA cultured in the presence of EGM and CGM (Fig. 1) clearly revealed that, compared to control cells, experimental cells contained significantly ($P < 0.05$) lower amounts of nonpolar lipid. Although the polar lipid contents of control and experimental cells were similar, the total lipid obtained by adding the nonpolar and polar lipid contents remained significantly ($P < 0.05$) different in both the PA (178 μg *vs.* 155 μg for control and experimental cells, respectively) and the DA (271 μg *vs.* 212 μg in control and experimental cells, respectively).

The contents and compositions of FA, as FAME, in control and experimental conditioned media on Days 0, 2 and 5 were determined and summed to obtain information on the FA remaining in the media from the 36 mL of original CGM and EGM used to culture the DA in these studies. These data are summarized in Table 5. As was observed for the original media, there were slightly more total FA in the experimental conditioned media. On a weight percent basis the distribution among the major FA was generally similar to that observed in the original growth media (Table 1). However, the conditioned media contained some FA that were not observed or were present in much lower proportions in the original growth media. These included 16:1*t* in the experimental conditioned

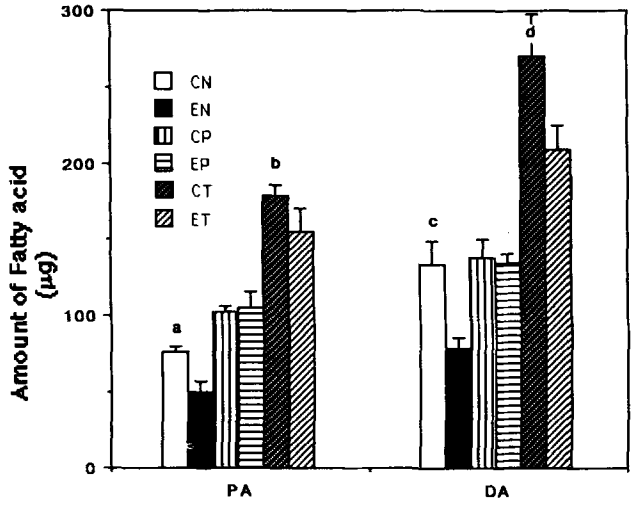

FIG. 1. Total fatty acids in polar and nonpolar lipids of 3T3-L1 cells cultured in the presence or absence of *trans* fatty acids. Bars represent means ± SEM for three independent determinations of total fatty acids/flask in polar, nonpolar and total lipids of preadipocytes (PA) and differentiating adipocytes (DA). CN, control nonpolar lipid; EN, experimental nonpolar lipid; CP, control polar lipid; EP, experimental polar lipid; CT, control total lipid = CN + CP; and ET, experimental total lipid = EN + EP. Significant differences ($P < 0.05$) between control and experimental cells are indicated by the letters a, b, c and d.

TABLE 5

Fatty Acid Content and Composition of Conditioned Media[a]

Fatty acid	Control		Experimental	
	μg	wt%	μg	wt%
14:0	30 ± 2	(0.5)	28 ± 0.9	(0.5)
16:0	766 ± 21	(13.3)	720 ± 11	(12.0)
16:1*c*	118 ± 5	(2.0)	97 ± 4	(1.6)
16:1*t*	ND		117 ± 3[b]	(2.0)
18:0	320 ± 17	(5.5)	431 ± 4[b]	(7.6)
18:1*c*	2672 ± 50	(46.3)	1680 ± 31[b]	(27.8)
18:1*t*	ND		780 ± 5[b]	(13)
18:2n-6	1723 ± 20	(30)	2074 ± 45[b]	(34)
18:3n-3	39 ± 2	(0.7)	40 ± 0.1	(0.7)
Others[c]	97 ± 3	(1.7)	90 ± 5	(1.5)
Total	5766 ± 52		6057 ± 54	

[a]Values are the means ± SEM for μg of fatty acid/flask (wt%) for three independent determinations of the total conditioned media obtained by summing values for media collected on Days 0, 2 and 5; ND, not detected; *c*, *cis*, *t*, *trans*.
[b]Denotes statistically significant differences between control and experimental media at $P < 0.05$.
[c]Includes fatty acids <0.5% (10:0, 12:0, 14:0, 15:0, 17:0 and 20:4n-6).

medium and 14:0 as well as 16:1*c* in both control and experimental conditioned media. Interestingly, these FA were observed in conditioned media in greater amounts than were found in the cultured cells. The presence of significant amounts of cellular FA in the conditioned media may have resulted from efflux of cellular lipids.

DISCUSSION

The 3T3-L1 cells readily incorporated exogenous FA, including *t*FA. Compared to PA, more *t*FA were incorporated in the polar and nonpolar lipids of DA, reflecting the longer contact of DA with the growth media. In polar lipids, *t*FA appeared to be incorporated at the expense of saturated FA. In particular, *t*FA tended to replace 18:0. This inverse relationship between *t*FA and saturated FA may be related to the similarity of their properties. Compared to *cis* FA, both *t*FA and saturated FA are linear molecules with relatively high melting points. Other similarities between *t*FA and saturated FA have been reported. These include the preferential incorporation of both types of FA into the 1-acyl position of phospholipids *in vivo* (8), the similar reactivities of these FA in acyl transferase reactions *in vitro* (9) and the similar responses to dietary intake of *t*FA or of saturated FA in raising the plasma level of LDL cholesterol (13,14) in humans.

Nevertheless, *t*FA have unique properties and should not be arbitrarily categorized together with saturated FA. Despite the apparently similar effects of *t*FA and saturated FA on LDL cholesterol, their effects on HDL cholesterol were reported (13) to be different. Unlike dietary *t*FA which decreased HDL cholesterol, saturated FA increased the level of this lipoprotein fraction. In our studies (Table 4), whereas *t*FA replaced saturated FA in the polar lipid fraction, this was not the case for the nonpolar lipid fraction, in which case *t*FA appeared to replace monounsaturated FA. A similar behavior of *t*FA was observed in studies of epididymal fat pads in C57Bl/6J mice (19). However, the functional significance, if any, of these biochemical observations cannot be ascertained from these studies.

Comparisons of FA in control and experimental cells suggested that *t*FA may have altered the metabolism of n-6 FA in experimental cells. The lower content of 20:4n-6 and higher ratio of 18:2n-6/20:4n-6 observed in the experimental cells is consistent with an effect of *t*FA on the conversion of 18:2n-6 to 20:4n-6; however, differences in the uptake of these FA or preferential catabolism or efflux of 20:4n-6 could also account for these observations. We are not aware of any reports of preferential uptake, catabolism or efflux of n-6 FA in tissues exposed to *t*FA. In contrast, *t*FA have been reported to inhibit desaturases in liver tissue (39) as well as in cell culture (40,41). In the cell culture studies with human skin fibroblasts, Δ9-18:1*t* was reported to be a potent inhibitor of Δ6 (40) and Δ5 (41) desaturase activities, both of which are important in the conversion of 18:2n-6 to 20:4n-6.

One of the most striking observations in this study was that, compared to cells exposed to CGM, 3T3-L1 cells exposed to EGM contained less total FA in the nonpolar lipid fraction. In contrast, the total FA level in the polar lipid fraction was similar in control and experimental cells. The similarity in polar lipid content and the difference in nonpolar lipid content suggested that fat accumulation, rather than the number of cells, may have been affected by the *t*FA in the EGM. This was further supported by the finding, in preliminary studies, that there were comparable numbers of cells and amounts of protein in both control and experimental flasks. The reason for the difference in fat accumulation is not known, but a lower availability of FA for experimental cells compared to control cells seems unlikely as a comparison of total FA values for CGM and EGM (Table 1) suggests that the experimental cells were exposed to a slightly higher concentration of FA in the growth medium than were the control cells.

It can be calculated that the experimental cells should have incorporated more FA than the control cells by comparing the data in Tables 1 and 5. The DA were exposed to the levels of FA shown in Table 1 on three occasions. Thus, on the average, control cells were exposed to 7356 μg, and experimental cells were exposed to 8556 μg of total FA. Since the average values for the total FA in the total conditioned media (Table 5) were 5766 and 6057 μg for control and experimental cells, respectively, the average amount taken up by control and experimental cells should have been 1590 and 2499 μg, respectively. These calculations suggest that uptake of FA was actually more, rather than less, in the experimental cells compared to the control cells, even though the resulting lipid of cells cultured in EGM was less than that of cells grown in CGM. Considering the total lipid available in the growth media, the lipid remaining in the conditioned media and the lipid accumulated in control DA (271 μg) and experimental DA (212 μg), more total lipid was unaccounted for in experimental cells (27%) than in the control cells (18%). These findings suggest that there was greater catabolism of FA in cells exposed to the EGM.

Compared to *cis* FA, many *t*FA have been reported to be less well oxidized by mitochondria (42–44). Nevertheless, the presence of significant amounts of 16:1*t* in the experimental conditioned media (Table 5) and in the experimental cells (Tables 2 and 3) provides evidence that these cells were capable of at least partially oxidizing 18:1*t*. Chain shortening of FA that are poorly oxidized by mitochondria has been postulated (42,45,46) to be an important function of the β-oxidation pathway in peroxisomes. Moreover, *t*FA appear to be capable of inducing peroxisomal proliferation. In studies with liver tissue isolated from C57Bl/6J mice (47), it was found that the number of peroxisomes and the level of cyanide-insensitive β-oxidation activity could be increased by dietary *t*FA. Others (42,48–50) have reported that a variety of dietary fats can cause induction of peroxisomal β-oxidation. It is possible that the decreased accumulation of lipid observed in the experimental cells in this study may have resulted from induction of peroxisomal β-oxidation by the *t*FA in the EGM.

Decreased accumulation of lipid in 3T3-L1 cells has been observed previously (20). In that study (20), the cells were treated with dibutyryl cAMP and epinephrine over a period of two weeks, and the reduction in lipid accumulation was attributed to increased activity of hormone sensitive lipase. Hormone sensitive lipase is unique to adipose tissue where stored triacylglycerols are degraded to glycerol and free fatty acids by this enzyme (51). If *t*FA increase peroxisomal β-oxidation activity in 3T3-L1 cells, it is likely that hormone-sensitive lipase may also be affected.

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Effect of Propylthiouracil-Induced Hypothyroidism on Membranes of Adult Rat Brain

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The effect of hypothyroidism on the lipid composition of myelin and synaptosomes isolated from adult rat brain was investigated. The animals were made hypothyroid by adding 0.05% propyl-2-thiouracil to their drinking water for four weeks. This pathological state resulted in a significant increase in the relative percentage of choline glycerophospholipids in synaptosomes with a concomitant decrease in ethanolamine glycerophospholipids as compared to controls. In myelin, hypothyroidism significantly influenced only the relative percentage of sulfatides. The effect of the hypothyroid state on mature brain was also reflected in changes in the membrane fatty acid composition. Myelin and synaptosomes showed an increase in arachidonic (20:4) and eicosatrienoic (20:3) acids and an increase in the fatty acid unsaturation index. Furthermore, the 20:4/20:3 and 20:3/18:2 ratios were lower and higher, respectively, in treated animals. The data indicate that hypothyroidism affects the lipid composition of synaptosomes and myelin even though the effects were less pronounced in myelin. The lipid changes observed in hypothyroidism may be of physiological significance, as it is well known that lipid composition modulates various membrane-bound enzymes, transporters and receptors. *Lipids 28,* **1075–1078 (1993).**

That a relationship exists between lipid metabolism and the thyroid state was first noted in 1931 by Wesson and Burr (1). Since then, many investigators have reported that hypothyroidism affects lipid content and composition of membranes isolated from different rat tissues and subcellular fractions (2–5). The fatty acid changes observed were interpreted as being consistent with a modulation of the activity of enzymes involved in their synthesis, desaturation and elongation (6–10). The mechanism responsible for producing the changes in membrane phospholipid (PL) composition is still not known.

Brain is an important target of thyroid hormones, with striking effects occurring during the development of the central nervous system (CNS). Hypothyroidism induced during the pre- and postnatal periods causes biochemical, morphological and behavioral changes (11,12). Alterations in brain lipid composition could be responsible for the observed changes in the activity of several synthesizing and degrading neurotransmitter enzymes (11).

The effect of hypothyroidism on mature brain is not well understood. A number of reports have recently indicated that thyroid hormone deficiency affects brain function in the adult rat (13–15), but data on biochemical changes were not reported.

In order to elucidate the effects of hypothyroidism on brain membranes, we studied the lipid composition of CNS myelin and of synaptosomes in adult rats with hypothyroidism induced by propylthiouracil (PTU).

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Wilmington, MA), weighing 200–210 g at the beginning of the experiments, were used. They were fed a standard diet *ad libitum.* The animals were made hypothyroid (HO) by adding PTU (0.05% wt/vol) to their drinking water for four weeks (16). They were then sacrificed, and the brains were quickly removed. Adequacy of HO state was assessed by measuring serum 3,3′,5,5′-tetraiodothyronine (T4) and 3,3′,5-triiodothyronine (T3) levels by radioimmunoassay (Serono Biodata, Milano, Italy). The PTU treatment led to a decrease in circulating mean T4 levels (21.6 ± 2.9 ng/mL), as compared with mean T4 value in euthyroid (EU) rats (88.5 ± 17.9 ng/mL; $P < 0.001$). A similar tendency was observed for the serum T3 levels. The respective mean values for T4 and T3 for six independent experiments were: 0.41 ± 0.06 ng/mL in HO and 0.95 ± 0.16 ng/mL in EU animals ($P < 0.01$).

Subcellular fractions. The brains were homogenized with a Dounce homogenizer in 0.32 M sucrose (5% wt/vol), and myelin was isolated by sucrose density gradient centrifugation according to Norton and Poduslo (17). Synaptosomes were prepared using a Percoll density gradient as suggested by Nagy and Delgado-Escuanta (18). The purity of both subcellular fractions was checked by electron microscopy. The membranes were submitted to osmotic shock with distilled water, pelleted by centrifugation, lyophilized and stored at −20°C.

Lipid analysis. Lipids were extracted from membranes with 20 vol of chloroform/methanol (2:1, vol/vol) according to Folch *et al.* (19). Lipids were separated into classes by one-dimensional high-performance thin-layer chromatography. In our solvent system, plasmalogens are not separated from the corresponding diacylglycerophospholipids. Lipids were quantified by *in situ* laser densitometry (model XL; Produckker, Bromma, Sweden) after staining with specific reagents for PL, cholesterol and galactolipids, as previously described (20). Peak areas were measured with an internal digital integrator. Lipids were analyzed for fatty acid composition by gas–liquid chromatography (GLC). The fatty acid methyl esters (FAME) were prepared by transmethylation with methanolic HCl (Supelco, Inc., Bellefonte, PA) at 70°C for 2 h and analyzed using a Hewlett-Packard HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector. Dimethylacetals derived from plasmalogens during the transmethylation procedure did not interfere with fatty acid analysis. This was ascertained on some samples when the fatty acid composition obtained was the same whether dimethylacetals were

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Abbreviations: CB, cerebrosides not containing hydroxy fatty acids; CBOH, cerebrosides containing hydroxy fatty acids; CPL, choline glycerophospholipids; CNS, central nervous system; EPL, ethanolamine glycerophospholipids; EU, euthyroid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; HO, hypothyroid; PL, phospholipids; PTU, propylthiouracil; PUFA, polyunsaturated fatty acids; T3, triiodothyronine; T4, tetraiodothyronine.

removed or not. Fatty acid composition was analyzed by GLC of the FAME at 260°C on a fused silica capillary column of Supelcowax 30 m × 0.53 mm i.d. (Supelco) using helium as carrier gas (flow rate 1.3 mL/min). Fatty acids were identified by comparison with commercial standards [polyunsaturated fatty acids (PUFA) 1 and PUFA 2; Supelco]. Quantitative data were derived using an integrator (Hewlett-Packard 3396A) and a standard mixture of FAME to establish the response factors for the components identified.

Statistical analysis. All results are expressed as means with standard deviations (SD). The statistical significance of mean differences was determined by Student's *t*-test.

RESULTS

The effect of PTU-induced hypothyroidism on the lipid composition of myelin is shown in Table 1. The relative percentage of each class of glycolipids was lower in the treated rats than in the controls. However, only the decrease in sulfatides was statistically significant. The ratio of hydroxy fatty acid containing cerebrosides (CBOH) to hydroxy fatty acid-free cerebrosides (CB) was increased in hypothyroidism (20%), mainly as the result of a more pronounced decrease in CB as compared to CBOH.

Table 2 shows the effect of thyroid hormone deficiency on the lipid composition of synaptosomes. A significant increase in the percentage of the choline glycerophospholipids (CPL) with a concomitant decrease in the ethanolamine glycerophospholipids (EPL) was observed in treated animals compared with controls. No differences were observed between the two groups in the other lipid classes.

The effect of PTU-induced hypothyroidism was also reflected in changes in myelin fatty acid composition. As shown in Table 3, the percentage of total monounsaturated fatty acids, which are characteristic fatty acids of myelin, is significantly lower in HO than in EU rats, mainly due to the decrease in 24:1 and 20:1. Total saturated fatty acids were not affected by the treatment, even though a significant decrease in 20:0 and 24:0 was observed. PUFA increased in both types of membranes of the treated rats. This increase was more pronounced in synaptosomes with a consequent elevation of the unsaturation index (Table 4). Because 20:3 increased more in synaptosomal membranes than did the other PUFA (50%), the 20:4/20:3 ratio decreased while the 20:3/18:2 ratio increased.

TABLE 1

Lipid Composition of Brain Myelin in Euthyroid and in Hypothyroid Rats[a]

Lipids[b]	Euthyroid rats	Hypothyroid rats
SPM	2.0 ± 0.56	2.4 ± 0.38
CPL	13.2 ± 1.07	13.5 ± 1.72
SPL + IPL	8.1 ± 2.53	8.3 ± 1.94
EPL	15.0 ± 3.13	15.7 ± 2.53
SULF	3.6 ± 1.23	2.2 ± 0.22[c]
CBOH	23.6 ± 4.37	22.0 ± 2.17
CB	7.7 ± 2.25	5.8 ± 0.74
CHOL	26.6 ± 6.01	30.3 ± 2.29
CBOH/CB ratio	3.1 ± 0.4	3.8 ± 0.31[c]

[a]Results are expressed as percent of total lipids and are given as means ± SD (n = 4).
[b]SPM, sphingomyelin; CPL, choline glycerophospholipids; SPL, serine glycerophospholipids; IPL, inositol glycerophospholipids; EPL, ethanolamine glycerophospholipids; SULF, sulfatides; CBOH, cerebrosides containing hydroxy fatty acids; CB, cerebrosides not containing hydroxy fatty acids; CHOL, cholesterol.
[c]$P < 0.05$.

TABLE 2

Lipid Composition of Brain Synaptosomes in Euthyroid and in Hypothyroid rats[a]

Lipids[b]	Euthyroid rats	Hypothyroid rats
SPM	2.8 ± 0.62	3.7 ± 1.01
CPL	34.6 ± 1.83	41.7 ± 4.62[c]
SPL + IPL	12.8 ± 0.82	14.9 ± 3.67
EPL	25.2 ± 2.40	18.2 ± 4.21[c]
CHOL	24.5 ± 2.30	21.4 ± 3.22

[a]Results are expressed as percent of total lipids and are given as means ± SD (n = 4).
[b]Abbreviations as in Table 1.
[c]$P < 0.05$.

TABLE 3

Fatty Acid Composition of Brain Myelin in Euthyroid and in Hypothyroid Rats[a]

Fatty acid	Euthyroid rats	Hypothyroid rats
16:0	7.5 ± 1.47	9.1 ± 0.41
16:1	0.4 ± 0.05	0.6 ± 0.00
18:0	19.0 ± 0.45	19.5 ± 0.42
18:1	33.3 ± 1.82	35.3 ± 0.4
18:2	0.6 ± 0.12	0.7 ± 0.12
20:0	2.1 ± 0.31	1.6 ± 0.06[b]
20:1	7.1 ± 1.18	5.7 ± 0.52[b]
20:3	0.7 ± 0.14	1.0 ± 0.12[b]
20:4	6.3 ± 0.56	7.8 ± 0.28[c]
22:0	2.4 ± 0.45	2.0 ± 0.09
22:1	0.8 ± 0.12	0.6 ± 0.19
23:0	0.8 ± 0.36	0.5 ± 0.06
22:4	2.7 ± 0.27	3.0 ± 0.30
24:0	4.7 ± 0.78	3.4 ± 0.46[b]
22:6	2.3 ± 1.97	2.5 ± 0.25
24:1	8.7 ± 2.03	6.5 ± 0.38[b]
Saturated	36.8 ± 1.44	36.2 ± 0.62
Monoenoic	50.4 ± 1.31	48.3 ± 0.48[b]
Polyenoic	12.7 ± 2.27	15.1 ± 0.7
UI[d]	103.2 ± 12.36	111.5 ± 2.57
20:4/20:3 ratio	9.3 ± 2.09	7.8 ± 2.1
20:3/18:2 ratio	1.0 ± 0.05	1.4 ± 0.00[b]

[a]Values are expressed as percent of total fatty acids and are given as means ± SD (n = 4).
[b]$P < 0.05$.
[c]$P < 0.005$.
[d]UI, unsaturation index, which is the sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds.

DISCUSSION

PTU-induced hypothyroidism affects the lipid composition both in myelin and synaptosomes from adult rat brain, even though the changes are more pronounced in the synaptosomes than in myelin. Synaptosomes of the HO rats show a significant increase in CPL with a concomitant decrease in EPL. Similar changes were shown to occur in heart mitochondria by Paradies and Ruggiero

TABLE 4

Fatty Acid Composition of Brain Synaptosomes in Euthyroid and in Hypothyroid Rats[a]

Fatty acids	Euthyroid rats	Hypothyroid rats
16:0	18.6 ± 2.47	11.9 ± 4.59[b]
16:1	1.2 ± 0.35	0.9 ± 0.5
18:0	25.0 ± 0.71	26.1 ± 1.35
18:1	23.5 ± 2.36	21.5 ± 1.71
18:2	1.6 ± 0.41	1.8 ± 0.55
20:0	0.3 ± 0.13	0.5 ± 0.31
20:1	1.3 ± 0.48	1.7 ± 0.59
20:3	0.6 ± 0.18	1.1 ± 0.23[b]
20:4	13.3 ± 1.24	16.7 ± 1.42[b]
22:4	2.6 ± 0.41	3.2 ± 0.76
22:5	0.5 ± 0.11	0.3 ± 0.06
22:6	11.4 ± 0.97	14.1 ± 4.49
Saturated	43.9 ± 2.46	38.5 ± 4.16[c]
Monoenoic	26.1 ± 2.43	24.0 ± 2.11
Polyenoic	30.0 ± 0.95	37.3 ± 6.12[b]
UI[d]	164.1 ± 5.74	197.3 ± 32.4[b]
20:4/20:3 ratio	21.6 ± 3.61	15.8 ± 3.81[c]
20:3/18:2 ratio	0.4 ± 0.06	0.6 ± 0.15[b]

[a]Values are expressed as percent of total fatty acids and given as means ± SD (n = 4).
[b]$P < 0.005$.
[c]$P < 0.05$.
[d]UI, unsaturation index, which is the sum of the percentage of each unsaturated fatty acid multiplied by the number of double bonds.

(3). By contrast, the PL composition of colonic apical plasma and erythrocyte membranes (2) were not significantly altered in the HO state. As the changes vary widely between cells and subfractions from various tissues, this may suggest that different membranes respond to thyroid hormone deficiency differently, depending on their function.

CPL can be synthesized by methylation of EPL (21,22), as well as by the cytidine diphosphocholine pathway (23). In brain the major route is the latter (*de novo* route); nevertheless, an increase in methyltransferase activity was observed in membranes rich in PUFA (24). Because a significant increase of PUFA is observed in synaptosomes of HO rats, we could suggest that this route could be operative in treated animals, resulting in an increase in CPL. Moreover, a higher percentage of PUFA may increase membrane fluidity. It is known that the successive methylation of EPL increases membrane fluidity, reducing its viscosity (21). On the other hand, modulation of insulin receptors and catecholamine has been shown to occur in hypo- and hyperthyroid rats (15). Recently, some changes in neurotransmitter metabolic activity were also observed in brain of hypothyroid adult rat (25,26). Therefore, it is tempting to suggest that one of the factors responsible for these changes could be the changes that occur in the lipid environment.

Our data show that in adult brain the thyroid hormones are also involved in PUFA metabolism, probably by modulating desaturase activities, as is known for other tissues (9). In synaptosomes the relative percentages of 20:3 and 20:4 are higher in HO than in EU rats; the increase in the 20:3/18:2 ratio and the decrease in the 20:4/20:3 ratio would indicate that Δ6 and Δ5 desaturases are affected differently by thyroid hormone deficiency. However, some factors other than desaturation activities may influence the fatty acid composition of HO rats, such as fatty acid synthesis, membrane lipid degradation and transport of fatty acids from one site to another.

The myelin membrane is more affected by thyroid hormone deficiency in the developmental period than at adult age. This different response could be due to regional differences in the distribution of receptors in adult brain. Their highest concentration was found in neuronal cell-rich regions, such as the amygdala, the hippocampus and the cortex, whereas the lowest concentration was found in the brain stem and in the cerebellum, which are myelin and oligodendroglial cell-rich regions (27–29). However, the decrease in relative monoenoic acid and sulfatide levels could affect membrane structural integrity and stability because these lipid components play an important role in maintaining myelin packing (30).

In conclusion, our data indicate that hypothyroidism affects the lipid composition of membranes isolated from adult rat brain, with synaptosomes being more susceptible than myelin.

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Action of the New Hypolipidemic Agent Lifibrol (K12.148) on Lipid Homeostasis in Normal Rats: Plasma Lipids, Hepatic Sterologenesis, and the Fate of Injected [^{14}C]Acetate

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Lifibrol, a new hypocholesterolemic agent with activity in humans, was examined in normal rats for its short-term and long-term effects on lipid homeostasis. Cholesterol (Chol) synthesis inhibition by lifibrol was demonstrated *in vitro* in liver minces from normal rats by following [1-^{14}C]acetate ([^{14}C]Ac) and DL-[2-^{14}C]mevalonate ([^{14}C]-MVA) incorporation into [^{14}C]Chol. When administered at 50 mg/kg/d, lifibrol reduced plasma total Chol and triglycerides (TG) ($P < 0.001$) within 24 h. The Chol reduction was largely a result of reduction of low density and very low density lipoprotein cholesterol (LDL + VLDL-chol) and a smaller decrease in high density lipoprotein cholesterol (HDL-chol). After 10 d, however, a rebound effect emerged, and after 41 d, plasma Chol, LDL + VLDL-chol, and HDL-chol were restored. In contrast, plasma TG remained at reduced levels ($P < 0.01$). The rebound is attributed to counter-regulation of hepatic sterologenesis that was assessed both *ex vivo* and *in vivo*. The *ex vivo* incorporation of [^{14}C]MVA and [^{14}C]octanoate into [^{14}C]Chol and total digitonin-precipitable [^{14}C]sterols ([^{14}C]DPS) in liver minces was increased 2- and 6-fold, respectively, in rats treated 6 d at 50 mg/kg. Similarly, *in vivo* incorporation of intraperitoneally injected [^{14}C]Ac into hepatic [^{14}C]DPS (2 h post-injection) was increased 2- to 5-fold at 50 mg/kg, and evidence for increased sterologenesis in nonhepatic tissue was also obtained. The increased hepatic sterologenesis, evident within 48 h, persisted out to 41 d of treatment by which time increases ($P < 0.002$) in hepatic Chol and carcass total sterols were observed. Additionally, incorporation of injected [^{14}C]Ac into hepatic [^{14}C]TG was inhibited 60% by lifibrol ($P < 0.001$), and the appearance of [^{14}C]TG in plasma was reduced. Circulating free [^{14}C]fatty acids ([^{14}C]FFA) were also reduced, but hepatic [^{14}C]FFA synthesis was unaffected, thus suggesting either a lesser release of newly formed FFA from liver or an enhanced removal from plasma.
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Lifibrol is a new hypolipidemic agent that reduces plasma total cholesterol (Chol) and low density lipoprotein cholesterol (LDL-chol) in experimental animals and humans (1–4), and in some species reduces plasma triglyceride (TG) levels also (1,3). Based upon *in vitro* studies in supernatants of rat liver homogenates, the putative mechanism of the Chol-lowering action of lifibrol is considered to be inhibition of hepatic Chol biosynthesis between acetate and β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) (1,2,5). In marmosets (1) and pigs (3) treated with lifibrol for 3 (40 and 100 mg/kg) or 6 (12.5, 25, 50 and 100 mg/kg) mon, respectively, dose-related plasma Chol reductions were sustained with no evidence for any development of counter-regulatory responses. Likewise, no indication of plasma Chol rebound was observed in human volunteers (mean % change from baseline) treated for 2 wk at up to 900 mg/d (4). However, in rats whose plasma Chol was lowered during a three-day treatment at 100 mg/kg (2), the incorporation of [1-^{14}C]acetate ([^{14}C]Ac) into digitonin-precipitable sterols was increased 3.7 times in homogenates of their livers. Since the stimulation of sterol synthesis could alter the long-range response, the experiments presented here were designed to provide a comprehensive examination of the response of rats to lifibrol over a time-course that extended to 41 d. Measurements were made of plasma lipid/lipoprotein levels [TG, total Chol, high density lipoprotein cholesterol (HDL-chol), and low density plus very low density lipoprotein cholesterol (LDL + VLDL-chol)], and hepatic sterologenesis was evaluated in liver minces *in vitro* and *ex vivo* using DL-[2-^{14}C]mevalonic acid ([^{14}C]MVA) and [^{14}C]Ac or [1-^{14}C]octanoate as precursors. Additionally, *in vivo* sterologenesis and other aspects of lipogenesis in liver and other organs were evaluated by following the fate of intraperitoneally injected [^{14}C]Ac 2h post-injection in 6 and 41 day-treated rats. The results indicate that lifibrol at 50 mg/kg significantly reduces plasma Chol and TG within 24 h of initiating treatment. However, counter-regulation sets in by 2 to 3 d as evidenced by 4-fold increases in hepatic sterologenesis that eventually lead to rebound of plasma Chol to normal levels or above. By contrast, plasma TG levels remain suppressed as long as drug is provided and are paralleled by decreased incorporation of [^{14}C]Ac into hepatic TG2h after intraperitoneal injection of the tracer. Evidence is also presented that liver and carcass sterol concentrations are significantly elevated in rats receiving 50 mg/kg/d for 41 d.

MATERIALS AND METHODS

Chemicals and supplies. The following materials were purchased from New England Research Products (Boston, MA): [^{14}C]Ac, sodium salt, sp. act. 56.0 Ci/mol; DL-[^{14}C]MVA, DBED salt, sp. act. 48.6 Ci/mol; [1-^{14}C]octanoic acid, sodium salt, 58.0 Ci/mol; Protosol tissue and gel solubilizer (NEF-935); and Liquefluor liquid scintillation counting cocktail (NEF-903). Caprylic acid, sodium salt (sodium octanoate) and digitonin were purchased from Sigma Chemical Company (St. Louis, MO). Silica Gel G-coated glass plates used for thin-layer chromatography (Sil G-25, 20 × 20 cm) were obtained from Brinkman Instruments Co. (Westbury, NY). Lifibrol (K12.148, 4-[4′-*tert*-butylphenyl]-1-[4′-carboxyphenoxy]-2-

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); Chol, cholesterol; HDL, high density lipoprotein; HMG-CoA, β-hydroxy-β-methylglutaryl-CoA; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.42); LDL, low density lipoprotein, TG, triglyceride; VLDL, very low density lipoprotein; [^{14}C]Ac, [1-^{14}C]acetic acid, sodium salt; [^{14}C]DPS, digitonin-precipitable [^{14}C]sterols; [^{14}C]FFA, free [^{14}C]fatty acid, [^{14}C]MVA, DL-[2-^{14}C]mevalonic acid, DBED salt.

butanol) was obtained from Klinge Pharma (Munich, Germany).

Animals and diets. Male TUC-Sprague-Dawley rats (Charles River Laboratories, Kalamazoo, MI) were used throughout the studies. The rats were individually housed in wire-bottom cages with free access to water and feed. All rats received Purina Rodent Chow No. 5002 (Purina Mills, St. Louis, MO) during a 7-d acclimation and were then continued on the diet or the diet admixed with lifibrol at a level calculated to provide the desired dose of drug based upon average daily feed consumption. Lifibrol administration had no negative effects on growth of the animals. In the longest study (41 d), terminal weights in the control group and 10 and 50 mg/kg lifibrol-treated groups were 391 ± 28, 390 ± 33 and 383 ± 25 g, respectively.

Injection and blood sampling procedures. All intraperitoneal injections and blood sampling were performed under diethyl ether anesthesia. Blood was drawn *via* cardiac puncture into heparinized syringes and placed on crushed ice for approximately 1 h until centrifugation at 10°C to separate the plasma.

Plasma lipid measurements. Plasma lipid measurements (total Chol, TG and HDL-chol) were performed with an Ektachem DT60 analyzer (Eastman Kodak, Rochester, NY) (6,7) and were completed within 2 h of plasma preparation. LDL + VLDL-chol was determined as the difference between total Chol and HDL-chol.

Lipid extraction from plasma and tissue samples. Plasma and tissue total lipid extracts were prepared by the method of Folch *et al.* (8) employing chloroform/methanol mixtures. The nonsaponifiable lipid fractions from liver and other tissues were obtained by *n*-hexane extraction following digestion/saponification of the samples for 45 min at 60°C in the presence of alcoholic KOH as detailed previously (9). Digitonin-precipitable sterols were obtained by redissolving nonsaponifiable lipid extracts in acetone/ethanol (1:1, vol/vol) and treating with digitonin for 18 h (10).

Thin-layer chromatography of total lipid extracts and nonsaponifiable lipid extracts. All thin-layer chromatography employed silica Gel G-coated glass plates that were developed with a running solvent consisting of *n*-hexane/diethyl ether/glacial acetic acid (146:50:4, by vol). This system was used to fractionate total lipid extracts into the various lipid classes (11) and to isolate Chol, lanosterol and squalene from nonsaponifiable lipid extracts (11).

Assay of sample radioactivity. In all instances, radioactivity was assayed in a liquid scintillation spectrometer (Beckman Instruments Inc., Irvine, CA; Model LS9800) in glass vials using 15 mL of Liquefluor (toluene-based) counting cocktail. Lipids isolated by thin-layer chromatography were scraped directly from the chromatoplates into the fluid-filled vials for assay (11). Digitonin-precipitable sterols were dissolved in pyridine prior to the addition of counting cocktail (12). Quench corrections were made using the external standardization method and counting efficiency for ^{14}C averaged 94%.

In vitro *and* ex vivo *hepatic sterologenesis studies.* The effect of lifibrol on hepatic sterologenesis was evaluated in liver minces from chow-fed rats (average weight 215 g) in the presence of exogenous lifibrol (*in vitro* studies) and in liver minces from rats (average weight 175 g) pretreated with the drug (50 mg/kg/d) for 6 d (*ex vivo* studies). Liver minces (500 mg) were prepared from samples of the central region of the large hepatic lobe as described previously (7,11) and were incubated at 37°C in 3.5 mL of Krebs-Ringer bicarbonate buffer, pH 7.4 (7,11) in the presence of appropriate ^{14}C-labeled substrates. In the *in vitro* studies, the incubations contained 2 μCi of either [^{14}C]MVA (DBED salt) or sodium [^{14}C]Ac. Lifibrol was added to the incubations, at the outset, dissolved in 12 μL of dimethylsulfoxide; control incubations received the vehicle alone. In the *ex vivo* experiments, the [^{14}C]MVA was present at 1 μCi/mL. Sodium [1-^{14}C]octanoate substituted for [^{14}C]Ac in order to avoid any probability of [^{14}C]Ac dilution resulting from possible alterations in the endogenous acetate pool size arising from drug treatment (13). The [^{14}C]octanoate was present at a level of 3 μCi/mL in the presence of 1 mM unlabelled sodium octanoate (13). After incubation, all samples were digested/saponified and their *n*-hexane extracts used for isolation of Chol on thin layers of Silica Gel G and total sterols by digitonin-precipitation as described above.

Intraperitoneal injections of [^{14}C]Ac. Rats (average weight 150 g) were randomly assigned into groups of 3 to 8 and treated with lifibrol for up to 41 d. In Study I (Table 2, shown later), the rats were treated for 0 (Controls), 1, 2, 3, 4 and 10 d (n = 3/group) at 50 mg/kg/d. Initiation of the drug was staged so that all groups were evaluated concurrently. In Study II (Table 2, shown later), the rats were treated for 41 d (Controls, n = 8; 10 mg/kg, n = 7; 50 mg/kg, n = 7) and were also evaluated concurrently. At the end of their treatment period, body weights were recorded (to be used in expressing whole body sterol estimates), and the rats were injected intraperitoneally with 20 μCi sodium [^{14}C]Ac. Two hours post-injection, 4.0 mL of blood was taken under ether anesthesia for plasma lipid analysis, and the rats were sacrificed by ether overdose. The carcasses and samples of liver from the large lobe were frozen at −20°C until processed further. In all of the rats, digitonin-precipitable [^{14}C]sterols ([^{14}C]DPS) were measured in the nonsaponifiable lipid extracts from liver. Additionally, in rats from the 41-day study, the incorporation of [^{14}C]Ac into the individual lipid classes was measured in total lipid extracts from plasma and liver.

Incorporation of intraperitoneally injected [^{14}C]Ac into Chol intermediates in nonhepatic tissue was also investigated 2 h post-injection in rats (average weight 150 g) that received lifibrol for 6 d (50 mg/kg/d). Squalene and lanosterol were isolated from the nonsaponifiable lipid fraction from kidney, spleen, testes and adrenal gland.

Carcass sterol analysis. All rats from the 41-day study were taken from the storage freezer, and the brains and brain stems were removed without thawing the carcasses. The carcasses were next placed into 2-L screw-cap polypropylene bottles that contained 1200 mL of 15% alcoholic KOH. The carcasses were permitted to digest for 5 d at room temperature and were shaken several times per day to facilitate tissue dissolution and dispersion. On the fifth day, the digests were placed on a magnetic mixer, and a large stirring bar was placed into each bottle. The digests were stirred for approximately 30 min; this permitted the final disintegration of the delipidized, decalcified bone residues. The digests were next transferred into 2-L volumetric flasks. The digestion bottles were rinsed clean by multiple washings with ethyl alcohol and the washings were transferred to the volumetric flask. The

samples were next filled up to the 2.0-L mark with ethyl alcohol. All steps beyond this point utilized standard quantitative analysis techniques employing volumetric glassware at all stages. Five-mL aliquots of the digests were extracted with *n*-hexane to recover the total sterol fraction. The extracts were evaporated to dryness under N_2, suspended in acetone/ethanol (1:1, vol/vol) and the sterols were precipitated with digitonin as above (10). The washed digitonide precipitates were assayed using the Lieberman-Burchard reaction (10). Digitonin-precipitable sterols in the carcasses were corrected upward by the sterol content of the 4.0-mL of blood drawn and the sample of liver that was removed for analysis prior to carcass digestion. This correction permitted calculation of total carcass digitonin-precipitable sterols (minus brain and brain stem, which were discarded). Digitonin-precipitation of sterols and their assay by color development with Lieberman-Burchard reagent (10) provides a quantitative measure of Chol in plasma, liver and most other tissues where all but traces of the total sterol are Chol. Skin, however, has numerous other sterols that would not be quantitatively measured by this methodology. Therefore, the presence of skin in the carcass analysis will result in a degree of underestimation of total carcass sterols.

Statistical analyses. All data are presented as mean ± SD. Statistical analyses were performed by one-way analysis of variance. Comparisons between groups were made using Student's *t*-test for unpaired variates. Differences were considered to be significant when P was equal to, or less than, 0.05.

RESULTS

In vitro *and* ex vivo *hepatic sterologenesis.* Confirmation of *in vitro* Chol synthesis inhibition by lifibrol was documented in normal rat liver minces as a prerequisite to undertaking the present studies. Exogenous lifibrol present at 10^{-4} and 10^{-3} M in the incubations reduced [^{14}C]Ac incorporation into [^{14}C]Chol by 23 ± 3% (n = 4) and 43 ± 18% (n = 4), respectively; the differences in the percentage inhibition between 10^{-3} and 10^{-4}, however, were not statistically different ($P > 0.05$). Lifibrol also inhibited the incorporation of [^{14}C]MVA into [^{14}C]Chol by 37% at 10^{-4}M (mean of n = 2; 46 and 28) and 64% at 10^{-3}M (mean of 2; 65 and 63).

The effect of lifibrol on sterologenesis was also evaluated *ex vivo* in liver minces from rats that were fed lifibrol in the diet for 6 d at doses of 15 and 50 mg/kg/d (Table 1); [1-^{14}C]octanoate and [^{14}C]MVA were used as precursors. No inhibition of sterologenesis was observed in either of the two treatment groups. To the contrary, the incorporation of both precursors into [^{14}C]DPS and [^{14}C]Chol was increased significantly ($P \leq 0.04$) in the 50 mg/kg group. This group also displayed a significant reduction in plasma Chol relative to the control group (64 ± 24 *vs.* 24 ± 3 mg/dL, $P < 0.04$). In contrast, rats that received the lower dose (15 mg/kg) registered no significant change in plasma Chol or in the incorporation of either precursor into [^{14}C]DPS or [^{14}C]Chol.

Effects of lifibrol on in vivo *hepatic lipogenesis, plasma lipids, and liver and carcass sterol content.* The *ex vivo* sterologenesis experiments described in Table 1 were followed up by studies of *in vivo* sterologenesis in rats that received lifibrol in the diet for 1, 2, 3, 4, 10 and 41 d at a dose of 50 mg/kg/d (Table 2). A 10 mg/kg dose was also included in the 41-day treatment. All rats received an intraperitoneal injection of [^{14}C]Ac and were killed 2 h post-injection.

The incorporation of [^{14}C]Ac into hepatic [^{14}C]DPS was increased significantly ($P < 0.05$) by 48 h after initiation of the 50 mg/kg dose. The increased incorporation persisted out to 41 d, at which time it exceeded the control values by greater than 4-fold; this would appear to be a maximal response since a similar increase was observed at 41 d in the 10-mg/kg group. Plasma lipid concentrations were also measured in the rats and demonstrated a rapid response to the hypolipidemic action of lifibrol (Study I, Table 2). Within the first 24 h after initiating treatment at 50 mg/kg, there were significant reductions ($P < 0.001$) of 60–70% in plasma total Chol and TG concentrations. HDL-chol was also reduced 37% ($P < 0.01$) during the same period. The plasma lipid fraction experiencing the greatest change, however, was the combined fraction of LDL + VLDL-chol, which was reduced by about 80% in 24 h. The plasma lipid-lowering effects of lifibrol were maximal at 4 d. Between 4 and 10 d, HDL-chol returned to control values; the other parameters stayed significantly below the control values but did show a definite "up-trend" toward control values. After 41 d of treatment at doses of 10 and 50 mg/kg (Table 2, Study

TABLE 1

Effect of Lifibrol (15 and 50 mg/kg for 6 d) on Plasma Cholesterol (mg/dL) and on the Incorporation of [1-^{14}C]octanoate and DL-[2-^{14}C]mevalonate into Cholesterol and Total Digitonin-Precipitable Sterols in Rat Liver MInces, *ex vivo* (dpm/g wet wt)[a]

Lifibrol daily dosage	Plasma total cholesterol (mg/dL)	[^{14}C]Octanoate		[^{14}C]Mevalonate	
		Chol	DPS	Chol	DPS
		(dpm/g wet wt)		(dpm/g wet wt)	
Control	64 ± 24	4962 ± 1743	4538 ± 1704	93252 ± 25026	108466 ± 19244
15 mg/kg	57 ± 21	3954 ± 819	3469 ± 848	92820 ± 20638	112929 ± 31556
50 mg/kg	24 ± 3[b]	34236 ± 24134[b]	33204 ± 21307[c]	158082 ± 24238[d]	210938 ± 13453[e]

[a]Liver minces (500 mg) prepared from rats (average weight 175 g) that received lifibrol in the diet for 6 d were incubated at 37°C for 90 min in 3.5 mL Krebs-Ringer bicarbonate buffer, pH 7.4, which contained 3 μCi/mL [1-^{14}C]octanoate or 1 μCi/mL DL-[2-^{14}C]mevalonate. [^{14}C]Cholesterol (Chol) and total digitonin-precipitable [^{14}C]sterols (DPS) were isolated from the nonsaponifiable lipid extracts of the tissues as detailed under Materials and Methods. Values are means ± SD of three animals/group.

[b-e]Statistically significantly different from the respective control mean ([b]$P < 0.04$; [c]$P < 0.03$; [d]$P < 0.015$; [e]$P < 0.001$).

TABLE 2

Plasma Lipid Concentrations and *in vivo* Hepatic Sterologenesis from Intraperitoneally Injected [1-^{14}C]Acetate in Rats Receiving Lifibrol for up to 41 Days[a]

Duration of lifibrol treatment (days, D)	N	Dose (mg/kg)	Plasma lipids (mg/dL)				Hepatic total digitonin-precipitable [^{14}C]sterols	
			Total Chol	Triglyceride	HDL-Chol	VLDL + LDL-Chol	(dpm/g liver wet wt)	(% control)
Study I								
Control	3	0	68 ± 2	101 ± 22	40 ± 2	32 ± 4	2940 ± 775	100
D-1	3	50	28 ± 2[b]	24 ± 11[b]	25 ± 1[c]	4 ± 3[b]	3900 ± 741	133
D-2	3	50	28 ± 9[b]	27 ± 5[b]	25 ± 8[c]	3 ± 2[b]	6687 ± 2291[e]	227
D-3	3	50	24 ± 8[b]	26 ± 4[b]	23 ± 10[c]	2 ± 1[b]	7553 ± 3056[d]	256
D-4	3	50	17 ± 3[b]	15 ± 8[b]	16 ± 3[b]	1 ± 1[b]	7687 ± 2997[d]	256
D-10	3	50	39 ± 9[b]	30 ± 11[b]	37 ± 8	2 ± 2[b]	5513 ± 1157	188
Study II								
Control	8	0	58 ± 10	109 ± 17	34 ± 5	24 ± 5	1070 ± 187	100
D41	7	10	89 ± 28[c]	60 ± 28[c]	57 ± 17[b]	32 ± 15	4787 ± 1764[b]	447
D-41	7	50	64 ± 20	55 ± 38[c]	44 ± 10	20 ± 14	4910 ± 2934[b]	459

[a]Rats receiving lifibrol in the diet for up to 41 d were killed 2 h after an intraperitoneal injection of 20 μCi sodium [1-^{14}C]acetate in 0.5 mL physiologic saline/ethanol (24:1, vol/vol). Plasma lipids were measured, hepatic nonsaponifiable lipids were obtained, and total digitonin-precipitable sterols were isolated as detailed under Materials and Methods. Values are means ± SD of the number of rats indicated by N. Abbreviations: Chol, cholesterol; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein. [b-e]Statistically significantly different from the respective control mean ([b]$P < 0.001$; [c]$P < 0.01$; [d]$P < 0.02$, [e]$P < 0.05$).

II), no hypocholesterolemic action was evident and VLDL + LDL-chol returned to control levels; the hypolipidemic action of lifibrol observed at this time was limited solely to its hypotriglyceridemic action (50% decrease in plasma TG, $P < 0.01$).

Hepatic total Chol and carcass total digitonin-precipitable sterols were also measured in the rats treated for 41 d with 10 and 50 mg/kg of lifibrol. Hepatic total Chol was increased significantly from a control mean of 1.97 ± 0.20 mg/g wet wt to 2.42 ± 0.20 ($P < 0.002$) and 2.31 ± 0.15 ($P < 0.002$) mg/g wet wt in the 10- and 50-mg/kg dose groups, respectively. Carcass total sterol (DPS) was also increased by lifibrol. Carcass DPS in the control group measured 1665 ± 103 mg/kg body weight compared with 1735 ± 96 and 1845 ± 100 mg/kg body wt in the 10- and 50-mg/kg groups, respectively. The increase at 50, but not at 10, mg/kg was significant ($P < 0.002$).

Injected [^{14}C]Ac was used not only to monitor sterol synthesis in liver (Table 2) but the synthesis of other major lipid classes as well [*i.e.*, free fatty acids (FFA), glycerolipids and cholesteryl esters] (Table 3). The hepatic synthesis of ^{14}C-labeled cholesteryl esters was increased two to three times in the treated rats. In contrast, synthesis of labeled TG was reduced about 60% ($P < 0.02$) by lifibrol

TABLE 3

Effect of Lifibrol on the *in vivo* Incorporation of Intraperitoneally Injected [1-^{14}C]Acetate into Hepatic and Plasma Free Fatty Acids and Esterified Lipids in Normal Rats After 41 Days of Treatment (10 and 50 mg/kg/d)[a]

Lifibrol daily dosage	N	Phospholipid	Triglyceride	Free fatty acid	Esterified cholesterol
		dpm/g liver wet wt			
Control	8	7764 ± 2728	2445 ± 1077	856 ± 293	161 ± 77
10 mg/kg	7	5042 ± 1436[b]	945 ± 312[c]	786 ± 340	532 ± 206[b]
50 mg/kg	7	5202 ± 1082[b]	961 ± 577[c]	663 ± 222	374 ± 292[d]
		dpm/mL plasma			
Control	8	2565 ± 497	326 ± 186	126 ± 55	165 ± 24
10 mg/kg	7	2487 ± 447	111 ± 86[e]	58 ± 21[e]	563 ± 292[c]
50 mg/kg	7	2437 ± 362	39 ± 29[f]	29 ± 21[f]	449 ± 195[d]

[a]Samples of liver and plasma from the 41-day rats described in Table 2 were extracted and fractionated by thin-layer chromatography as detailed under Materials and Methods. Values are means ± SD of the number of rats, indicated by N.
[b-f]Statistically significantly different from the control mean ([b]$P < 0.03$; [c]$P < 0.001$; [d]$P < 0.02$; [e]$P < 0.001$; [f]$P < 0.003$).

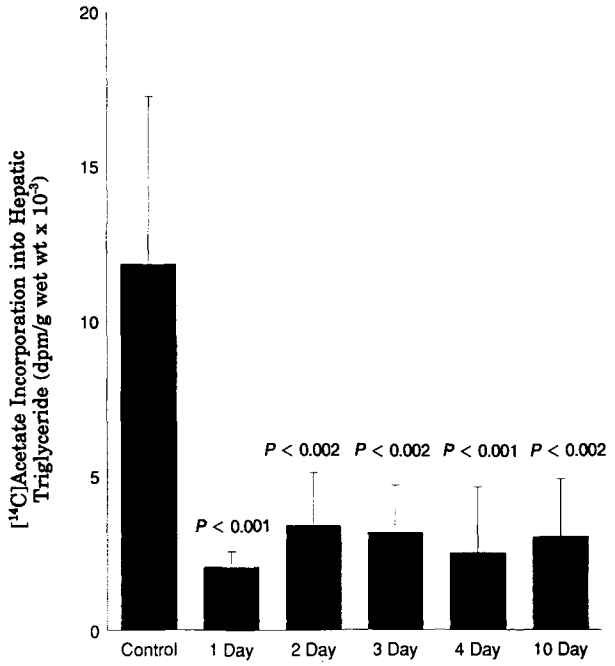

FIG. 1. Effect of lifibrol on the incorporation of intraperitoneally injected [1-^{14}C]acetate into hepatic triglycerides in normal rats. Rats (initial average weight 150 g) which received lifibrol (50 mg/kg/d) in the diet for up to 10 d were injected intraperitoneally with 20 μCi [1-^{14}C]acetate 2 h prior to killing. Samples of liver were extracted and fractionated by thin-layer chromatography to obtain the triglyceride fractions that were assayed for radioactivity as detailed under Materials and Methods. Values shown are means ± SD of three rats per group.

treatment. A smaller but significant reduction (approximately 35%, $P < 0.02$) in labeled phospholipid synthesis also occurred with both doses of lifibrol. These changes occurred in the absence of any significant differences in the amount of ^{14}C-labeled FFA recovered from the livers. The impact of lifibrol on hepatic TG synthesis has a rapid onset as shown by the data in Figure 1. Rats that received 50 mg/kg showed an 80% decrease in hepatic TG synthesis within 24 h of receiving treatment with lifibrol. The data of Figure 1 and Table 3 give no indication that the rats become refractory to the hypotriglyceridemic action.

Lipid newly synthesized from [^{14}C]Ac was also present in the plasma of the rats (Table 3). The profile of the labeled plasma lipid in the lifibrol-treated rats differed from the controls in two major respects. The first of these was a significant ($P < 0.003$) dose-related lowering of circulating ^{14}C-labeled TG and FFA; these changes occurred in the absence of any drug-related effect on the levels of circulating ^{14}C-labeled phospholipids. The second relates to the cholesteryl esters. Just as with liver, the plasma from treated rats had higher levels of ^{14}C-labeled cholesteryl esters (dpm/mL).

Relative sterologenesis activity in nonhepatic tissues. Evidence for an effect of lifibrol on *in vivo* sterologenesis in nonhepatic tissue (kidney, spleen, testes and adrenal glands) was investigated 2 h after intraperitoneal injection of [^{14}C]Ac into untreated rats (control, n = 6) and rats treated with lifibrol for 6 d (50 mg/kg, n = 6) (Table 4). Although [^{14}C]Chol was found in each of the tissues, its presence in the plasma (1814 ± 747 and 538 ± 286 dpm/mL in the lifibrol *vs.* control group, respectively, $P < 0.003$) precluded any attempt to relate tissue [^{14}C]Chol to *in situ* synthesis. However, no [^{14}C]squalene or [^{14}C]-lanosterol could be detected in the plasmas from either group. It was considered, therefore, that the presence of these Chol intermediates in nonhepatic tissues could be taken as an indication of *in situ* sterologenesis. The ratio of [^{14}C]squalene or [^{14}C]lanosterol recovered (dpm/g) in treated *vs.* control rat tissues provides an index of relative differences in sterologenesis activity. On this basis, the data indicate that lifibrol treatment increased the relative rate of synthesis about 2-fold (Table 4).

DISCUSSION

The studies presented here clearly demonstrate that the hypocholesterolemic effects of lifibrol in normal rats are

TABLE 4

Estimated Relative Rates of Sterol Synthesis in Nonhepatic Tissues from Rats Receiving Lifibrol (50 mg/kg/d) for Six Days[a]

	[^{14}C]Squalene			[^{14}C]Lanosterol		
	Lifibrol	Control	Ratio	Lifibrol	Control	Ratio
	(dpm/g wet wt)			(dpm/g wet wt)		
Kidney	148 ± 109	68 ± 12	2.2	188 ± 77[b]	67 ± 17	2.8
Spleen	35 ± 34[c]	49 ± 82	0.7	188 ± 86[c]	103 ± 61	1.8
Testes	62 ± 17[d]	40 ± 13	1.6	136 ± 35[e]	77 ± 10	1.8
Adrenal gland	45 ± 29[f]	17 ± 10	2.6	14 ± 14[c]	4 ± 7	3.5

[a]Rats (average weight 150 g) that received lifibrol in the diet (50 mg/kg) for 6 d were killed 2 h after intraperitoneal injection with 20 μCi sodium [1-^{14}C]acetate in 0.5 mL physiologic saline/ethanol (3:2, vol/vol). The organs were removed and fractionated by thin-layer chromatography to obtain the cholesterol intermediates, [^{14}C]squalene and [^{14}C]lanosterol, as detailed under Materials and Methods. The mean ± SD recovery of labeled lanosterol and squalene (dpm/g wet wt) in each tissue (n = 6 per group) was expressed as a ratio of lifibrol *vs.* control. Since these labeled intermediates of cholesterol were not detected in plasma, their presence in the tissues was taken as evidence for active *in situ* sterologenesis. The assumption is that their amount recovered from the tissues is proportional to the rate of sterologenesis extant.

[b-e]Statistically significantly different from the corresponding control mean ([b]$P < 0.004$; [c]not significant; [d]$P < 0.04$; [e]$P < 0.003$, [f]$P < 0.05$). Plasma total cholesterol was 47 ± 9 and 25 ± 4 mg/dL ($P < 0.0001$) in the lifibrol *vs.* control group, respectively, and [^{14}C]acetate incorporation into hepatic [^{14}C]cholesterol was 38232 ± 23843 *vs.* 6710 ± 2398 dpm/g ($P < 0.009$), respectively.

transient and not sustainable with extended treatment (Table 2). This differs from the experiences in marmosets and pigs where the hypocholesterolemic effect of the drug persists throughout treatment periods of 3 to 6 mon (1,3). Analysis of lipoproteins in pigs receiving 50 mg/kg lifibrol yielded results similar to the rats studied here (Ref. 3; Table 2). The overall lowering of plasma Chol involved reductions in HDL-chol in both species but was largely accounted for by lowering of the LDL + VLDL-chol. In the case of pigs, it has been proposed that lifibrol lowers the LDL through increased hepatic LDL-receptor activity which is the response to reduced hepatic Chol synthesis (2,3). Inasmuch as this is a reasonable hypothesis for the pig, the response of rats would appear to be more complicated since reduced plasma LDL + VLDL-chol and increased hepatic sterologenesis are observed concurrently during the hypocholesterolemia phase in the rat (Tables 1 and 2).

The stimulation of hepatic sterologenesis in the rat begins within 48 h of lifibrol treatment and persists as long as drug is administered (Table 2). This increased synthesis likely accounts for the frank rebound in plasma Chol observed at 41 d and indicated as early as 10 d (Table 2). Data from the *ex vivo* liver mince studies in lifibrol-treated rats (Table 1) and *in vivo*-treated rats injected with [1-^{14}C]acetate (Table 2) suggest that the increased hepatic sterologenesis is a function of dose and time. Doses of 50 mg/kg, but not 15 mg/kg, increased *ex vivo* hepatic sterologenesis in 6-day treated rats, whereas treatment for 41 d at similar or higher doses (10 and 50 mg/kg) yielded equivalent increases in sterologenesis (4.5-fold, Table 2).

Clearly, the intrinsic ability of lifibrol to inhibit sterologenesis as demonstrated here, using [^{14}C]Ac and [^{14}C]-MVA, and elsewhere (1) is readily neutralized in normal rats over time. The species difference in tolerance to lifibrol may be related to differences in the degree of counter-regulation achievable or to differences in the site(s) of drug action. In this regard, Schliak *et al.* (2) have proposed that the sustained hypocholesterolemia induced by lifibrol in pigs argues for inhibition of sterol synthesis prior to the rate-limiting, HMG-CoA reductase (14) step, whereas inhibition of [^{14}C]MVA incorporation into hepatic sterols *in vitro* in rat liver minces in the presence of exogenous lifibrol described here suggests that post-reductase site(s) of inhibition also exist (*i.e.*, assuming no drug-induced changes in substrate access). The reductions in sterol synthesis in liver minces cannot be attributed to an overall inhibition of lipogenesis since [^{14}C]Ac incorporation into total saponifiable lipid (dpm/g wet wt) is either unchanged or slightly increased in the presence of drug (9480 $\pm$ 1942, 11866 $\pm$ 2770, and 15648 $\pm$ 3544 at 0, 10^{-4} and 10^{-3} M lifibrol respectively, n = 3/group). More definitive studies will be necessary to more specifically characterize the inhibitory sites of lifibrol in the liver and the actions which determine counter-regulation. The relatively small increases in inhibition of sterol synthesis from [^{14}C]Ac and [^{14}C]MVA that we observed in going from 10^{-4} to 10^{-3} M lifibrol in liver minces suggests that factors such as drug solubility and tissue uptake may be limiting in some *in vitro* systems.

Another important observation made in these studies relates to the effect of lifibrol on tissue sterol concentration. In the 41-day rat study, hepatic Chol concentration was increased significantly at 10 and 50 mg/kg; carcass total sterols were also increased significantly at 50 mg/kg. The accumulation of body sterol may be a direct consequence of overproduction of sterol not only by the liver, but by certain nonhepatic tissues as well (Table 4).

Apart from increased sterologenesis, the most steadfast response of the normal rat to lifibrol was the hypotriglyceridemic response. A 24-hour treatment period at 50 mg/kg was sufficient to significantly lower plasma TG by 76% (Table 2). At the same dose, Schliak *et al.* (3) reported plasma TG lowering in male (but not female) pigs. However, the onset of the reduction was much slower to develop and was not statistically significant until some time between 2 and 12 wk. Unlike the situation with plasma Chol, there is no strong indication that plasma TG lowering in the rat is transient in nature. Quite to the contrary, it is a direct reflection of the reduced TG synthesis in the liver (Table 3 and Fig. 1) and the subsequent reduced appearance of newly synthesized TG in plasma (Table 3) that was demonstrated in the [^{14}C]Ac injection studies. The fact that the 41-day treated rats maintained significant reduction in plasma TG even though VLDL + LDL-chol were not significantly different from control levels (Table 2) suggests that the hypotriglyceridemia is not secondary to problems in LDL production and/or secretion. Future studies of VLDL secretion and turnover will more specifically address this issue.

The inhibition of TG synthesis appers to be relatively specific in that phospholipid synthesis was only modestly affected and fatty acid synthesis was not significantly affected (Table 3). It was observed, however, that newly formed ^{14}C-labeled FFA circulating in plasma were significantly lower in 41-day lifibrol-treated rats (Table 3). Whether or not this effect, which seems to be dose-dependent, represents a lesser release from liver (or other tissues) or a drug-enhanced clearance by peripheral tissues is not known. In the studies presented here, we did not assay directly the activity of plasma LCAT (lecithin/Chol acyltransferase; EC 2.3.1.42) or hepatic ACAT (acylCoA/Chol acyltransferase; EC 2.3.1.26), which are the main determinants of the status of plasma and hepatic cholesteryl esters (15). It is therefore possible that the higher levels of ^{14}C-labeled cholesteryl esters found in plasma and liver of the 41-day treated rats reflect lifibrol-induced increases in the activity of these enzymes. Alternately, the increase may reflect the availability of higher specific radioactivity Chol in both compartments as a result of increased synthesis of hepatic [^{14}C]Chol.

At the time of this writing, it is not known to what extent the long-term response of animals to lifibrol will be reflective of the long-term response in humans. In a 14-day human volunteer trial, lifibrol at 300, 600 and 900 mg/d significantly decreased plasma total Chol and LDL-chol by up to 30% or more without significantly affecting plasma HDL-chol and TG (4). These results, which were similar to the response in pigs given somewhat higher doses for 6 mon (3), gave no indication of an impending rebound in plasma Chol. In fact, plasma Chol and LDL-chol were still in a definite downslope after 2 wk, indicating that maximal lowering, at the doses provided, had not been reached. Provided that long-term safety and efficacy of lifibrol can be demonstrated clinically, the drug may be useful in the management of hypercholesterolemia.

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Effects of Lovastatin on Hepatic Fatty Acid Metabolism

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The *in vitro* and *in vivo* effects of lovastatin on fatty acid metabolism were studied in isolated rat hepatocytes. When added *in vitro* to cell incubations, lovastatin stimulated *de novo* fatty acid synthesis and acetyl-CoA carboxylase activity, whereas fatty acid synthase activity was unaffected. Lovastatin depressed palmitate, but not octanoate, oxidation. This may be attributed to the lovastatin-induced increase in intracellular malonyl-CoA levels, as no concomitant change of carnitine palmitoyltransferase I (CPT-I) specific activity was detected. Lovastatin had no effect on the synthesis and secretion of triacylglycerols and phospholipids in the form of very low density lipoproteins (VLDL). When rats were fed a diet supplemented with 0.1% (w/w) lovastatin for one week, both acetyl-CoA carboxylase activity and *de novo* fatty acid synthesis were reduced compared to pair-fed controls, whereas fatty acid synthase activity was unaffected. Palmitate oxidation was enhanced in the lovastatin-fed group. There was an increase in CPT-I activity but no change in intracellular concentration of malonyl-CoA. Lovastatin feeding had no significant effect either on the esterification of exogenous palmitic acid into both cellular and VLDL triacylglycerols and phospholipids or on hepatic lipid accumulation. The *in vitro* and *in vivo* effects of lovastatin were not significantly different between periportal and perivenous hepatocytes. The results indicate that: (i) the administration of lovastatin increased the fatty acid-oxidative capacity of the liver at the expense of its lipogenic capacity, (ii) the rate of *de novo* cholesterol synthesis did not seem to be a limiting factor in the synthesis and secretion of VLDL and (iii) lovastatin produced opposite effects on hepatic fatty acid metabolism *in vitro* and *in vivo*.
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The liver plays a central role in the synthesis and redistribution of cholesterol (1,2), and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase catalyzes the rate-limiting step in hepatic *de novo* cholesterol synthesis (3-5). The amounts and activities of hepatic HMG-CoA reductase are controlled very closely by a series of mechanisms acting at the transcriptional, translational and post-translational levels (3-5).

Increased plasma low density lipoprotein (LDL)-cholesterol levels are recognized as a major risk factor in the development of atherosclerosis and coronary heart disease (6,7). With the design of nontoxic inhibitors of HMG-CoA reductase, and therefore of cholesterol biosynthesis, treatment of hypercholesterolemic patients has become more effective (8-10). Among the different inhibitors of HMG-CoA reductase, lovastatin (mevinolin) is one of the most widely used agents, owing to its high efficacy and low toxicity (8-10). Inhibition of liver cholesterol biosynthesis increases the number of apo B/apo E (LDL) receptors on the hepatocyte surface, and this in turn causes lower plasma concentrations of LDL-cholesterol (8-12). By contrast, plasma levels of anti-atherogenic high density lipoprotein-cholesterol are unaffected or even raised by lovastatin treatment (8-10). Administration of lovastatin to animals and humans has been shown to affect bile acid metabolism as well (13,14).

Much less is known about the effects of lovastatin on hepatic fatty acid metabolism. It is well recognized that the regulation of cholesterol and fatty acid biosynthesis appear intertwined; *i.e.*, in physiopathological situations in which cholesterol biosynthesis is increased, fatty acid biosynthesis seems also to be enhanced, and *vice versa* (5,15,16). HMG-CoA reductase and acetyl-CoA carboxylase, the rate-limiting enzyme of *de novo* fatty acid synthesis, are phosphorylated, and thus inactivated, by the same protein kinase, namely the adenosine monophosphate-activated protein kinase (5,17). Treatment of rats with lovastatin leads to an induction of hepatic phosphatidate phosphohydrolase, one of the putative regulatory enzymes of triacylglycerol synthesis (18). In addition, a close relationship exists in rat liver between HMG-CoA reductase and the key regulatory enzyme of long-chain fatty acid oxidation, *viz.* carnitine palmitoyltransferase I (CPT-I) (19,20). Thus, inhibition of CPT-I leads to an induction of HMG-CoA reductase (19), whereas prolonged lovastatin feeding increases CPT-I activity (20). Hence, it is likely that lovastatin administration may induce important changes in hepatic fatty acid metabolism. In addition, by inhibiting cholesterol synthesis in the liver and/or by acting on fatty acid-metabolizing pathways, lovastatin has been shown to interfere with hepatic synthesis of lipoproteins. Thus, it has been reported that chronic treatment with lovastatin decreases very low density lipoprotein (VLDL) secretion in perfused rat liver (21), as well as in the intact animal (22,23). More recently, a similar effect of lovastatin administration has been shown to occur in humans (24). In addition, treatment with lovastatin and other HMG-CoA reductase inhibitors resulted in reduced plasma VLDL levels (8-10,22-24).

In the present article we report on the *in vitro* and *in vivo* effects of lovastatin on fatty acid metabolism in rat hepatocytes. Because different pathways of lipid metabolism are operative at different rates in the periportal and perivenous zones of the liver acinus (25), we also looked at possible differences in the observed effects of lovastatin on periportal and perivenous hepatocytes.

MATERIALS AND METHODS

Materials. ^3H_2O (5 Ci/mol), [1-^{14}C]acetyl-CoA (54 Ci/mol), [U-^{14}C]palmitic acid (403 Ci/mol), [1-^{14}C]palmitic acid (58 Ci/mol), [1-^{14}C]octanoic acid (32 Ci/mol) and L-[*methyl*-^{14}C]carnitine (54 Ci/mol) were supplied by Amersham International (Amersham, Bucks, United Kingdom). Lovastatin was kindly furnished by Dr. J. González Esteban (Merck, Sharp & Dohme, Madrid, Spain). Digitonin, bovine serum albumin (fraction V; essentially fatty acid-free) and collagenase (type I) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were analytical reagent grade from Sigma, Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany) and Aldrich (Steinheim, Germany).

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Abbreviations: ATP, adenosine triphosphate; CPT-I, carnitine palmitoyltransferase I; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoproteins; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

Animals. Male Wistar rats (225–275 g body weight) were used in all experiments. The *in vitro* effects of lovastatin were studied in hepatocytes isolated from rats fed a standard, stock-pelleted diet (21% of total calories as protein, 15% as fat and 64% as carbohydrates). The *in vivo* effects of lovastatin were studied in hepatocytes isolated from rats pair-fed, for one week, the same standard diet either supplemented (lovastatin group) or not (control group) with 0.1% (w/w) lovastatin. Animals were housed individually and kept in a constant-temperature room with a 12-h light–dark cycle (light, 6 a.m. to 6 p.m.; dark, 6 p.m. to 6 a.m.) Hepatocytes were isolated at 9 a.m. Food intake and body weight gain were not significantly different in lovastatin-fed and control animals (results not shown).

Isolation of hepatocytes. Hepatocytes from the whole liver were isolated as described by Beynen *et al.* (26), whereas periportal and perivenous hepatocytes were isolated by the digitonin/collagenase perfusion technique of Chen and Katz (27), and further characterized based on several marker enzyme activities (25,28). As lipogenesis is markedly depressed just after the isolation procedure, hepatocytes were incubated for 20 min at 37°C in a metabolic gyratory shaker and then filtered through nylon mesh (25,26). The final hepatocyte preparation was suspended in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose. Cell viability, as determined by Trypan Blue exclusion, always exceeded 85% in the final hepatocyte suspension.

Hepatocyte incubations. Hepatocytes were incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 2% (wt/vol) defatted and dialyzed bovine serum albumin. Incubations were carried out in a metabolic gyratory shaker (85 rpm) in a total volume of 2 mL at 37°C in an O_2/CO_2 (19:1) atmosphere. Cell concentration was adjusted to 4–6 mg of cellular protein per mL (1.3–2.1 $\times$ 10^6 cells/mL) in order to obtain comparable rates of fatty acid metabolism independent of cell density (25,28). For the study of the *in vitro* effects of lovastatin, stock solutions of lovastatin were prepared in dimethylsulfoxide. Control incubations contained corresponding dimethylsulfoxide levels; no significant effect of dimethylsulfoxide on fatty acid metabolism was observed at the concentrations used (0.2%, vol/vol).

Rates of fatty acid metabolism and cholesterol synthesis. Hepatocytes were incubated as described earlier to monitor the rates of *de novo* fatty acid and cholesterol synthesis, fatty acid esterification and fatty acid oxidation. The three parameters were determined at time points when reaction rates were linear (results not shown).

The rates of *de novo* fatty acid and cholesterol synthesis were determined by following the incorporation of ^3H_2O into total fatty acids and total cholesterol, respectively. Reactions were initiated by the addition of ^3H_2O (0.4 mCi/mL) to the hepatocyte incubations. After 60 min, the reactions were stopped, and lipids were extracted as described previously (29). Total lipids were saponified at 75°C with 0.3 M NaOH in 90% (vol/vol) methanol. After acidification of the samples, fatty acids and cholesterol were extracted with petroleum ether (b.p. 40–60°C) (30). Cholesterol was separated from the other lipid fractions by thin-layer chromatography (TLC) on silica gel G plates using hexane/diethyl ether/formic acid (40:20:1, by vol) as the developing system.

The rates of fatty acid esterification were monitored by following the incorporation of [U-^{14}C]palmitic acid into major lipid classes. Hepatocytes were incubated in the presence of 0.4 mM albumin-bound [U-^{14}C]palmitic acid (0.05 Ci/mol), and after 60 min, cells were transferred into centrifuge tubes and separated from the medium (31) from which VLDL were isolated by gradient ultracentrifugation (32). The appearance of VLDL in the incubation medium showed a lag phase of *ca.* 20 min. Therefore, rates of VLDL secretion were calculated between 30 and 60 min of cell incubation. Cellular and VLDL lipids were extracted (29) and subsequently separated by TLC on silica gel G plates using hexane/diethyl ether/formic acid (40:20:1, by vol) as the developing system. Individual bands were scraped off for determination of radioactivity.

The rates of fatty acid oxidation were measured by following the formation of oxidation products from either [1-^{14}C]palmitate or [1-^{14}C]octanoate. Hepatocytes were incubated in the presence of 0.4 mM albumin-bound [1-^{14}C]fatty acid (0.05 Ci/mol), and after 30 min, the reactions were stopped by addition of 0.5 mL of 2 M perchloric acid. At the same time, 0.1 mL of 6 M KOH was injected into a center well containing filter paper. Samples were allowed to equilibrate for an additional 60 min, and the content of the center well (with the $^{14}CO_2$ fixed as bicarbonate) was transferred to vials for radioactivity counting. Ketone bodies were determined as the nonvolatile, acid-soluble oxidation products (33). Total oxidation products were calculated as the sum of CO_2 plus ketone bodies.

Acetyl-CoA carboxylase assay. The activity of acetyl-CoA carboxylase was determined by following the incorporation of radiolabelled acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction, both in the 12000 $\times$ *g* supernatant (29) and in digitonin-treated hepatocytes (30). This method does not suffer from interferences, as does the classical bicarbonate-fixation assay of acetyl-CoA carboxylase activity (30). In order to measure enzyme activity in digitonin-treated hepatocytes, 100 μL of hepatocyte suspension were added to 100 μL of digitonin-containing assay medium. The final assay mixture contained 63 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (pH 7.5), 1.5 mM $MgCl_2$, 0.5 mM citrate, 2.5 mM ethyleneglycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, 22.5 mM $NaHCO_3$, 70.5 mM NaCl, 5 mM glucose, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 2 mM adenosine triphosphate (ATP), 0.5 mM NADPH, 0.44 mM dithioerythrol, 0.93% bovine serum albumin (defatted and dialyzed), 0.062 mM [1-^{14}C]acetyl-CoA (4 Ci/mol), 0.062 mM butyryl-CoA, 64 μg of digitonin/mg of cellular protein and 3.2 milliunits of rat liver fatty acid synthase. After 4 min, reactions were stopped with 0.1 mL of 10 M NaOH and fatty acids were extracted and radioactivity was measured (30).

Fatty acid synthase assay. The activity of fatty acid synthase was determined using digitonin-treated hepatocytes and by following the incorporation of radiolabelled acetyl-CoA into fatty acids under conditions in which acetyl-CoA carboxylase remains inactive (30). One hundred μL of hepatocyte suspension were added to 100 μL of digitonin-containing assay medium. The final assay mixture contained the same components as the acetyl-CoA carboxylase assay, except that butyryl-CoA was replaced by malonyl-CoA and ATP; citrate and purified fatty acid synthase were omitted. After 8 min, reactions

were stopped with 0.1 mL of 10 M NaOH, and fatty acids were extracted and radioactivity measured (30).

CPT-I assay. The activity of CPT-I was determined by following the malonyl-CoA-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine, both in isolated mitochondria and in digitonin-treated hepatocytes. In the first case, mitochondria were isolated from intact hepatocytes, and CPT-I activity was measured as described previously (34). Preparations of mitochondria were practically devoid of peroxisomes, as judged by measuring catalase activity (results not shown).

In order to determine CPT-I activity in digitonin-treated hepatocytes (35), 100 μL of hepatocyte suspension were added to 100 μL of digitonin-containing assay medium. The final assay mixture contained 12.5 mM Tris-HCl (pH 7.4), 70 mM sucrose, 5 mM glucose, 32.5 mM KCl, 12.5 mM $NaHCO_3$, 60 mM NaCl, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1 mM ethylenediaminetetraacetic acid, 1 mM dithioerythrol, 50 μM palmitoyl-CoA, 0.5 mM L-[*methyl*-^{14}C]carnitine (1 Ci/mol), 0.5% bovine serum albumin (defatted and dialyzed) and 40 μg of digitonin/mg of cellular protein. After 1 min, reactions were stopped with 0.3 mL of 1 M HCl and [^{14}C]palmitoylcarnitine product was extracted with *n*-butanol as described before (33).

In both the isolated mitochondria and the digitonin-treated hepatocyte assays, malonyl-CoA-insensitive CPT activity, representing the latent form of mitochondrial CPT activity (CPT-II), was always subtracted from the total CPT activity that was experimentally determined. CPT-I activity always accounted for more than 90% of this total CPT activity. Furthermore, the degree of inhibition of hepatocellular CPT activity by 100 μM malonyl-CoA was similar within a wide range of palmitoyl-CoA concentrations (20-200 μM) (results not shown).

Other analytical procedures. Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method using purified rat liver fatty acid synthase (26). Kits from Boehringer were used for the determination of cellular cholesterol and triacylglycerols.

Statistical analysis. Results shown represent the means ± SD of the number of animals indicated. Cell incubations and/or enzyme assays were carried out in triplicate. Statistical analysis was performed by the Student's *t*-test.

RESULTS

In vitro *effects of lovastatin.* The *in vitro* effects of lovastatin on fatty acid metabolism were studied in isolated rat hepatocytes. In preliminary experiments, lovastatin was shown to decrease *de novo* cholesterol synthesis in a dose-dependent manner (Fig. 1). A similar trend was observed with regard to the appearance of labelled VLDL cholesterol in the extracellular medium (93 ± 5% inhibition in the presence of 0.1 mM lovastatin). A lovastatin concentration of 0.1 mM was chosen as standard condition.

Addition of lovastatin to the hepatocyte incubation increased the rate of *de novo* fatty acid synthesis by 35% (Table 1). This increase correlated well with the lovastatin-mediated stimulation of acetyl-CoA carboxylase activity, as measured in digitonin-treated cells (Table 1). No

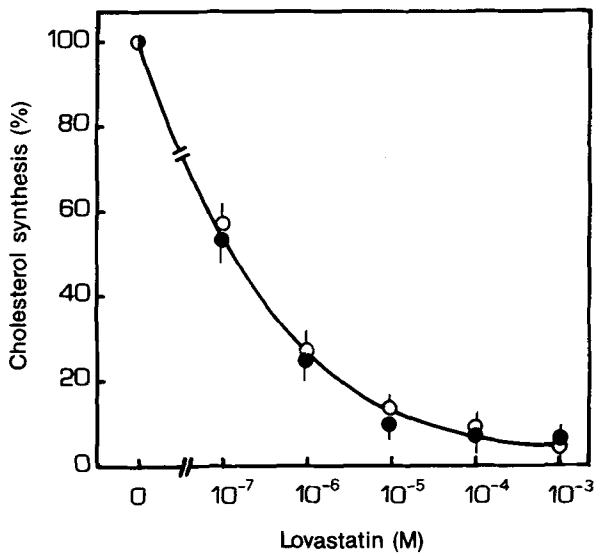

FIG. 1. ***In vitro*** **effect of lovastatin on hepatic** ***de novo*** **cholesterol synthesis. Periportal (○) and perivenous (●) hepatocytes were preincubated for 30 min in the presence of different concentrations of lovastatin and** ***de novo*** **cholesterol synthesis was followed. Results represent the means ± SD of three different preparations of periportal and perivenous hepatocytes. The 100% level for the rate of cholesterol synthesis (in nmol acetyl units/h × mg cell protein) was 10.54 ± 2.31 and 6.47 ± 1.26 in periportal and perivenous hepatocytes, respectively.**

direct effect of lovastatin was observed on acetyl-CoA carboxylase activity, as measured in the 12000 × *g* supernatant (results not shown). In addition, lovastatin showed no effect on fatty acid synthase activity (Table 1).

In cell incubations, lovastatin had no significant effect on the rate of fatty acid esterification from exogenous palmitate into the major glycerolipid fractions, namely triacylglycerols and phospholipids (Table 1). Isolated hepatocytes have previously been used to study the secretions of newly synthesized VLDL lipids (1,28,36). Thus, we monitored the rate of VLDL lipid secretion into the incubation medium by following incorporation of exogenous palmitic acid into the VLDL fraction. This approach is valid as long as changes in fatty acid incorporation into cellular lipids are also considered. When cells were incubated with [^{14}C]palmitic acid, no effect of lovastatin was observed on the appearance of labelled VLDL triacylglycerols and VLDL phospholipids in the extracellular medium (Table 1).

The rate of palmitate oxidation was depressed by the addition of lovastatin to the incubation medium; the decrease was seen in both the CO_2 and the acid-soluble component of total oxidation products (Table 1). However, lovastatin had no significant effect on hepatic octanoate oxidation (Table 1). As can be seen in Table 1, CPT-I activity was not changed when hepatocytes were incubated with lovastatin. In addition, no direct effect of lovastatin was observed on CPT-I activity measured in isolated mitochondria (results not shown).

Inhibition of CPT-I activity by malonyl-CoA is a well-described property of the enzyme (16,37-39). Hence, we determined the effects of lovastatin on hepatic malonyl-CoA levels. In line with the lovastatin-mediated stimulation of acetyl-CoA carboxylase activity previously

TABLE 1

***In vitro* Effects of Lovastatin on Hepatic Fatty Acid Metabolism[a]**

Metabolic parameter	Additions to the incubations: None	Additions to the incubations: 0.1 mM Lovastatin
Fatty acid synthesis	33.6 ± 4.7	45.9 ± 3.5[b]
Palmitate esterification		
Cell lipids		
Triacylglycerols	8.65 ± 1.34	7.96 ± 0.52
Phospholipids	6.49 ± 0.68	5.45 ± 0.59
VLDL lipids		
Triacylglycerols	0.94 ± 0.12	0.88 ± 0.05
Phospholipids	0.24 ± 0.07	0.26 ± 0.06
Palmitate oxidation		
Acid-soluble product	42.1 ± 6.9	24.4 ± 5.0[c]
CO_2	5.2 ± 0.6	3.5 ± 0.4[b]
Total product	47.3 ± 7.7	27.9 ± 3.3[c]
Octanoate oxidation		
Acid-soluble product	97.3 ± 10.2	108.0 ± 13.1
CO_2	11.3 ± 1.8	11.5 ± 2.6
Total product	108.6 ± 9.7	119.5 ± 15.4
Enzyme activities		
Acetyl-CoA carboxylase	0.60 ± 0.07	0.75 ± 0.04[b]
Fatty acid synthase	1.65 ± 0.20	1.68 ± 0.07
CPT-I	1.53 ± 0.18	1.62 ± 0.32

[a]Hepatocytes were isolated from whole liver and preincubated in the absence or in the presence of 0.1 mM lovastatin. After 30 min, part of the cells was used for determination of the rates of fatty acid synthesis, esterification [in both cell and very low density lipoproteins (VLDL) lipids] and oxidation in intact hepatocytes. The rest of the cells were used for measurement of enzyme activities in digitonin-treated hepatocytes. Rates of fatty acid synthesis are expressed as nmol acetyl units/h × mg cell protein. Rates of fatty acid esterification and oxidation are expressed as nmol fatty acid into product/h × mg cell protein. Enzyme activities are expressed as nmol product/min × mg cell protein. Results represent the means ± SD of six hepatocyte preparations. CoA, coenzyme A; CPT-I, carnitine palmitoyltransferase I.

[b]$P < 0.05$ between incubations with no additions and lovastatin-containing incubations.

[c]$P < 0.01$ between incubations with no additions and lovastatin-containing incubations.

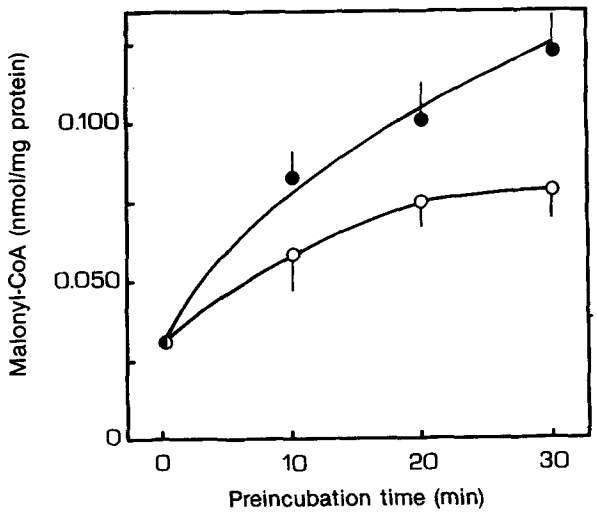

FIG. 2. *In vitro* effect of lovastatin on hepatocyte levels of malonyl-CoA. Hepatocytes isolated from whole liver were preincubated for 30 min in the absence (○) or in the presence (●) of 0.1 mM lovastatin, and intracellular levels of malonyl-CoA were subsequently determined. Results represent the means ± SD of three different hepatocyte preparations. CoA, coenzyme A.

mentioned here, lovastatin induced a significant increase in the intracellular concentration of malonyl-CoA in intact hepatocytes (Fig. 2).

Because HMG-CoA reductase is predominantly located in the periportal zone of the liver (40), the aforementioned *in vitro* effects of lovastatin on hepatic fatty acid metabolism were studied in periportal and in perivenous hepatocytes. However, no significant differences between the two hepatocyte subpopulations were found with regard to the effects of lovastatin on fatty acid synthesis and oxidation. Thus, lovastatin stimulated *de novo* fatty acid synthesis by 42 ± 10% in periportal hepatocytes and by 31 ± 11% in perivenous hepatocytes, whereas lovastatin increased acetyl-CoA carboxylase activity by 24 ± 4% in periportal hepatocytes and by 27 ± 5% in perivenous hepatocytes. In addition, lovastatin depressed palmitate oxidation to CO_2 by 30 ± 8% in periportal hepatocytes and by 47 ± 7% in perivenous hepatocytes.

In vivo *effects of lovastatin.* The *in vivo* effects of lovastatin on fatty acid metabolism were studied in hepatocytes isolated from rats that had been pair-fed a standard diet either supplemented (lovastatin group) or not (control group) with 0.1% (w/w) lovastatin. In preliminary experiments, we observed that the rate of *de novo* cholesterol synthesis in hepatocytes isolated from lovastatin-treated animals was 8.4 ± 2.1-fold higher than in control cells. This may be due to the well-described induction of HMG-CoA reductase upon prolonged lovastatin administration (3,40).

Both the activity of acetyl-CoA carboxylase and the rate of *de novo* fatty acid synthesis were depressed in cells from lovastatin-fed animals, whereas fatty acid synthase activity was not significantly changed (Table 2).

Lovastatin administration had no significant effect on the esterification of exogenous [^{14}C]palmitic acid into cellular triacylglycerols and phospholipids (Table 2). In addition, the rate of appearance of labelled VLDL triacylglycerols and VLDL phospholipids into the extracellular medium was not affected by lovastatin administration (Table 2). The lipid content of hepatocytes isolated from lovastatin-fed animals was similar to that of pair-fed controls (Table 3).

The rates of hepatic palmitate oxidation to both CO_2 and acid-soluble products were increased in hepatocytes isolated from lovastatin-treated animals as compared to control cells (Table 2). A similar trend was observed with regard to CPT-I activity (Table 2). As most of the long-term variations of hepatic long-chain fatty acid oxidation are accompanied by changes in malonyl-CoA levels (16,37-39), hepatocytes were isolated from the two animal groups and the intracellular concentration of malonyl-CoA was determined. However, no significant differences in malonyl-CoA concentrations were detected. The resulting values (in nmol/mg cell protein) were 0.038 ± 0.016, 0.036 ± 0.009, 0.048 ± 0.013 and 0.038 ± 0.007 for the control group periportal zone, control group perivenous zone, lovastatin group periportal zone and lovastatin group perivenous zone, respectively.

Induction of HMG-CoA reductase by administration of lovastatin or cholestyramine appears to occur

TABLE 2

In vivo Effects of Lovastatin on Hepatic Fatty Acid Metabolism[a]

Metabolic parameter	Animal group	
	Control	Lovastatin-fed
Fatty acid synthesis	27.0 ± 3.5	13.1 ± 2.3[b]
Palmitate esterification		
Cell lipids		
Triacylglycerols	7.92 ± 1.78	8.05 ± 2.67
Phospholipids	7.41 ± 1.35	8.39 ± 1.80
VLDL lipids		
Triacylglycerols	0.85 ± 0.27	0.67 ± 0.21
Phospholipids	0.25 ± 0.07	0.25 ± 0.09
Palmitate oxidation		
Acid-soluble product	44.3 ± 7.9	62.1 ± 9.6[b]
CO_2	5.8 ± 2.8	8.2 ± 1.4[c]
Total product	50.1 ± 8.2	70.3 ± 10.8[b]
Enzyme activities		
Acetyl-CoA carboxylase	0.48 ± 0.07	0.17 ± 0.05[b]
Fatty acid synthase	1.26 ± 0.68	1.46 ± 0.71
CPT-I	1.31 ± 0.17	1.82 ± 0.16[b]

[a]Hepatocytes from whole liver were isolated from animals fed either the control or the lovastatin-containing diet for one week. Part of the cells were used for determination of the rates of fatty acid synthesis, esterification (into both cell and VLDL lipids) and oxidation in intact hepatocytes. The rest of the cells were used for measurement of enzyme activities in digitonin-treated hepatocytes. Rates of fatty acid synthesis are expressed as nmol acetyl units/h × mg cell protein. Rates of palmitate esterification and oxidation are expressed as nmol palmitate into product/h × mg cell protein. Enzyme activities are expressed as nmol product/min × mg cell protein. Results represent the means ± SD of eight different animals of each group. Abbreviations as in Table 1.
[b]$P < 0.01$ between control and lovastatin-fed animals.
[c]$P < 0.05$ between control and lovastatin-fed animals.

predominantly in the perivenous zone of the liver (40). Hence, we determined whether the effects of lovastatin feeding on hepatic fatty acid synthesis and oxidation differed between periportal and perivenous hepatocytes. The lovastatin-induced reduction of fatty acid synthesis (and of acetyl-CoA carboxylase activity), and the lovastatin-induced increase in palmitate oxidation (and CPT-I activity) were seen equally in both liver zones. Thus, lovastatin administration depressed *de novo* fatty acid synthesis by 55 ± 8% in periportal hepatocytes and by 47 ± 9% in perivenous hepatocytes, whereas it reduced acetyl-CoA carboxylase activity by 56 ± 11% in periportal hepatocytes and by 65 ± 8% in perivenous hepatocytes. In addition, lovastatin administration enhanced palmitate oxidation to CO_2 by 39 ± 14% in periportal hepatocytes and by 45 ± 18% in perivenous hepatocytes. It also increased palmitate oxidation to acid-soluble products by 32 ± 13% in periportal hepatocytes and by 48 ± 15 in perivenous hepatocytes, and enhanced CPT-I activity by 41 ± 17% in periportal hepatocytes and by 37 ± 13% in perivenous hepatocytes.

DISCUSSION

The *in vitro* and *in vivo* effects of lovastatin on fatty acid metabolism were studied in rat hepatocytes with a twofold goal. On the one hand, lovastatin is known to be useful in modulating the rates of hepatocellular cholesterol synthesis, thus allowing study of the relationship between cholesterol synthesis and fatty acid metabolism both *in vitro* and *in vivo*. On the other hand, lovastatin is widely used in clinical practice and, hence, we considered it of interest to investigate both the rapid response (*in vitro* studies) and the prolonged effects (*in vivo* studies) of administering to animals.

The parallel *in vitro* stimulation of acetyl-CoA carboxylase activity and *de novo* fatty acid synthesis by lovastatin supports the general view that acetyl-CoA carboxylase is a key regulatory point in the fatty acid-synthesizing process (5,15). Malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase, is a physiological inhibitor of CPT-I and plays an essential role in the coordinate control of fatty acid synthesis and oxidation in the liver (37-39). It is well established that palmitate is transported into the mitochondria by a carnitine-dependent process, whereas octanoate may enter mitochondria independently of carnitine (16,37-39). Therefore, the lovastatin-induced increase of malonyl-CoA levels, together with the differential effects of lovastatin on palmitate and octanoate oxidation in intact hepatocytes, indicates that decreased CPT-I activity might be involved in the lovastatin-mediated inhibition of long-chain fatty acid oxidation. Other components of the fatty acid-translocation system, namely acyl-CoA synthetase, carnitine:acylcarnitine translocase and CPT-II, are generally not considered to play a significant regulatory role in the transport of long-chain fatty acids into the mitochondrial matrix (16,37-39). Although we were not able to detect any rapid effect of lovastatin on CPT-I activity, it should be pointed out that, in order to measure

TABLE 3

Lipid Content of Hepatocytes Isolated from Control and Lovastatin-Fed Rats[a]

Lipid fraction	Control group		Lovastatin-fed group	
	PP (n = 4)	PV (n = 5)	PP (n = 5)	PV (n = 4)
Triacylglycerols	57.0 ± 8.4	63.7 ± 14.0	63.2 ± 9.0	60.8 ± 14.8
Cholesterol				
Free	42.9 ± 13.2	49.3 ± 10.5	46.1 ± 10.0	39.4 ± 3.9
Esterified	53.4 ± 7.8	52.5 ± 9.8	60.2 ± 12.1	56.5 ± 8.7
Total	96.3 ± 18.7	101.8 ± 12.1	106.3 ± 21.8	95.9 ± 8.9

[a]Cellular levels of triacylglycerols, free cholesterol and esterified cholesterol were determined in periportal (PP) and perivenous (PV) hepatocytes isolated from control and lovastatin-fed animals. Lipid content is expressed as nmol lipid/mg cell protein. Results represent the means ± SD of the number of hepatocyte preparations indicated in parentheses.

enzyme activity, we had to make the plasma membrane permeable. This may have caused the cytosol to leak out of the cell, leading to a substantial dilution of cytosolic components, including malonyl-CoA. Lovastatin is not expected to induce an irreversible, *e.g.*, covalent, modification of CPT-I protein. Conformational constraints exerted by malonyl-CoA on CPT-I disappear almost immediately at the temperature at which the assay is carried out (37°C) (37). Therefore, if lovastatin exerts rapid changes in CPT-I activity, they will probably not be preserved in digitonin-treated hepatocytes. It is thus possible that increased malonyl-CoA levels would inhibit CPT-I activity in intact hepatocytes, but not in digitonin-treated hepatocytes. As a matter of fact, short-term changes in CPT-I activity induced by a number of agonists, such as hormones and phorbol esters, seem to involve changes in the phosphorylation state of the enzyme, which may be preserved upon digitonin treatment of the plasma membrane (35,41,42).

We realize that the *in vitro* effects of lovastatin on fatty acid synthesis, oxidation and esterification seem somewhat contradictory. Thus, if fatty acid synthesis is increased and oxidation is reduced, there should be an increase in the incorporation of labelled palmitate into lipids. Reduced oxidation of the tracer might be balanced by additional synthesis of unlabeled palmitate. However, in palmitate-containing incubations, the fatty acid oxidized or esterified is mostly of exogenous origin. We have observed that addition of 0.5 mM palmitic acid to the hepatocyte incubations strongly blunts fatty acid synthesis through inhibition of acetyl-CoA carboxylase, as determined in digitonin-treated hepatocytes. Although hepatic lipogenesis and long-chain fatty acid oxidation usually change in concert (*i.e.*, higher rates of lipogenesis are accompanied by lower rates of oxidation, and *vice versa*), there are situations in which this relationship is not seen (see Ref. 39). The reasons for these discrepancies are not known.

Changes in hepatic acetyl-CoA carboxylase activity observed in lovastatin-fed animals did not parallel changes in malonyl-CoA content. However, this is not the only case where this occurs. For example, during lactation (43), or after prolonged ethanol feeding (29,44), acetyl-CoA carboxylase activity decreases but malonyl-CoA levels remain unaltered. This may be due to the fact that malonyl-CoA levels depend not only on changes in the activity of acetyl-CoA carboxylase, but also on variations in the activity of a number of malonyl-CoA consuming enzymes, such as fatty acid synthase, malonyl-CoA decarboxylase and the microsomal fatty acid elongation/desaturation system (30,37). Because the rate of palmitate oxidation was enhanced in the lovastatin-fed group, this would indicate that the intracellular concentration of malonyl-CoA, the physiological inhibitor of CPT-I activity, is not the factor responsible for the regulation of hepatic long-chain fatty acid oxidation under these conditions. Increased long-chain fatty acid oxidation after lovastatin administration seems to involve, at least in part, an increase in CPT-I activity (see also Ref. 20). Other examples of malonyl-CoA independent control of hepatic long-chain fatty acid oxidation have been described previously (41,43-46). Although changes in rat liver CPT-I activity usually occur in concert with changes in enzyme sensitivity to inhibition by malonyl-CoA (37-39), the latter parameter has been shown to remain unaltered by lovastatin treatment (20). Something similar is known to occur after prolonged treatment of rats with other drugs, such as anabolic steroids (47).

It is worth noting that lovastatin caused opposite effects *in vitro* and *in vivo* on hepatic fatty acid synthesis and oxidation. Upon addition of lovastatin to the incubation medium, *de novo* cholesterol synthesis was blocked, and cytosolic acetyls may be diverted into fatty acid synthesis at the expense of cholesterol synthesis. However, in the long term it is well known that lovastatin produces a very strong induction of the HMG-CoA reductase enzyme (3,40). In the whole animal the continuous presence of lovastatin in the diet ensures that hepatic HMG-CoA reductase activity (and thus cholesterol biosynthesis) is permanently blunted in spite of the increase in enzyme concentration (9,10). By contrast, in hepatocytes isolated from lovastatin-fed animals, *de novo* cholesterol synthesis is markedly enhanced because of the induction of HMG-CoA reductase and the absence of lovastatin in the extracellular medium (3,40). Therefore, cytosolic acetyls would preferentially be used for cholesterol synthesis at the expense of fatty acid synthesis. Hence it seems that fatty acid and cholesterol synthesis compete for the same cytosolic acetyl-CoA pool.

On the basis of *in vitro* and *in vivo* studies, Khan *et al.* (21,22) have suggested that cholesterol availability is a regulatory factor for the secretion of VLDL by the liver. In our system, addition of lovastatin to the hepatocyte incubation medium strongly decreased both cholesterol synthesis *de novo* and the secretion of newly synthesized cholesterol as VLDL. However, lovastatin had no effect on the secretion of newly synthesized VLDL triacylglycerols and VLDL phospholipids. Likewise, Khan *et al.* (21) did not observe any significant effect of lovastatin on VLDL output by perfused rat liver when the drug was directly added to the perfusion medium, although lovastatin strongly decreased *de novo* cholesterol synthesis from [^{14}C]acetate when either control or lovastatin-fed animals were used as liver donors. Moreover, in the present study, we showed that hepatocytes isolated from lovastatin-fed rats displayed an enhanced capacity for *de novo* cholesterol synthesis and for secretion of this newly synthesized cholesterol, but they secreted VLDL triacylglycerols and VLDL phospholipids at the same rate as cells isolated from control animals. Therefore, the rate of *de novo* cholesterol synthesis did not seem to be a limiting factor for VLDL secretion by the liver, at least in the experimental system employed herein. This does not seem surprising, as the total pool of hepatic cholesterol (free plus esterified) would be very large compared to the metabolic pool required for the formation and secretion of VLDL (21).

Intermediary metabolism, including fatty acid metabolism, is asymmetrically distributed within the liver acinus (25,27,48-50). However, no differences between periportal and perivenous hepatocytes have been noted in the short-term modulation by cellular effectors of lipid (25) and carbohydrate (51) metabolism. This was also the case in short-term incubations with lovastatin. In addition, the zonal heterogeneity of hepatic intermediary metabolism is flexible and may change during the development of the animal and upon physiopathological changes, including starvation, diabetes and alcohol ingestion (28,48-50,52).

By contrast, the distribution of fatty acid metabolism within the liver acinus remains unchanged after lovastatin administration. The situation *in vivo*, of course, must be more complicated due to the existence of concentration gradients for nutrients, hormones and oxygen, as well as due to the particular innervation of the two domains of the liver acinus (48–50). Ongoing studies are directed toward defining the effects of lovastatin on fatty acid metabolism in the whole animal.

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Depletion of Adipose Tissue and Peripheral Nerve α-Tocopherol in Adult Dogs

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To assess the relationship between tissue α-tocopherol depletion and histopathologic or functional changes in nervous tissue, a longitudinal study of male 1-year-old beagle dogs, two fed a vitamin E-deficient diet (0.05 ± 0.02 mg α-tocopherol/kg; −E dogs) and two fed a vitamin E-supplemented diet (114 ± 14 mg α-tocopherol/kg; +E dogs), was carried out. Plasma and adipose tissue α-tocopherol concentrations, neurological examinations, and sensory and motor nerve conduction velocities were determined at approximately 8-wk intervals over 109 wk. Tibial nerve α-tocopherol concentrations were measured at 65 and 109 wk; adjacent sections were examined for histologic changes. In the two −E dogs, plasma α-tocopherols declined linearly on a semilog plot to < 0.1 μg/mL by 109 wk. Plasma α-tocopherol concentrations were depleted to half of the initial concentrations in approximately 87 d. Adipose tissue α-tocopherol concentrations (based on wet weight, cholesterol or triglyceride) also declined linearly on semilog plots, and were depleted to half of the initial concentrations in approximately 120 d. Tibial nerve α-tocopherols (ng/μg cholesterol) in −E dogs decreased to 16% of average +E at 65 wk, and to 2% at 109 wk. Neurologic examinations, histologies and nerve conduction velocities were normal in all dogs throughout the study. Our results demonstrate in dogs that depletion of plasma, adipose tissue and nerve α-tocopherol precedes histologic and functional changes in peripheral nerves during vitamin E deficiency.

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Symptoms of vitamin E deficiency in humans and experimental animals include spinocerebellar ataxia and peripheral neuropathy (for review, see Ref. 1); therefore, vitamin E deficiency should be considered in the diagnosis of patients with neurological disease. Measurements of plasma α-tocopherol (α-T) concentrations have been widely used for assessment of vitamin E status, but are not necessarily the most reliable indicator because plasma α-T values vary with lipid levels (2).

Adipose tissue α-T can be a more reliable indicator of vitamin E status than plasma concentrations. For example, adipose tissue α-T levels are depleted in vitamin E-deficient humans (3), while normal adipose tissue α-T levels are associated with the prevention of neurologic dysfunction in patients at risk for vitamin E deficiency, consuming adequate levels of vitamin E supplements (4). In response to supplementation with vitamin E, adipose tissue α-T in most species do increase (5–10). Further, adipose tissue α-T concentrations are correlated with sural nerve α-T concentrations in humans (3).

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Abbreviations: α-T, α-tocopherol; −E, dogs fed a vitamin E-deficient diet, +E, dogs fed a vitamin E-supplemented diet; i.m., intramuscularly; NCV, nerve conduction velocity; SD, standard deviation; $t_{1/2}$, time to deplete half of initial concentrations; TG, triglycerides.

Longitudinal studies in rodents demonstrated that, in response to a vitamin E-deficient diet, adipose tissue α-T is conserved compared with that in other tissues (5,6). However, rodents may not be accurate models of human vitamin E deficiency because they develop clinical signs of the deficiency within months, as opposed to humans, in whom clinical signs can take several years to decades to develop (1).

The purpose of this study was to compare tissue α-T depletion with functional and histopathologic changes in nervous tissue. Beagle dogs were chosen as an alternative animal model of experimental vitamin E deficiency because they have a longer life span than rodents. Vitamin E deficiency in dogs has been documented to cause pathologic changes in smooth and skeletal muscles, central nervous system and retina, as well as hematologic changes (11,12). The dogs were fed semi-purified casein-based diets with or without (−E, dogs fed a vitamin E-deficient diet; +E, dogs fed a vitamin E-supplemented diet) added vitamin E for 109 wk. At approximately 8-wk intervals, we performed physical and neurologic examinations, measured plasma and adipose tissue α-T, and sensory and motor nerve conduction velocities (NCV). We collected biopsy (65 wk) and necropsy (109 wk) samples of the tibial nerve to determine the depletion of α-T from nerves in comparison with adipose tissue, and to evaluate histopathologic changes.

MATERIALS AND METHODS

Protocol. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee (Auburn, AL). Four 1-year-old male beagle dogs (Marshall Farms, North Rose, NY), weighing 18 kg, were fed a purified diet containing *all-rac*-α-tocopheryl acetate (TD No. 87238; Teklad Laboratories, Madison, WI; α-tocopherol, 114 ± 14 mg/kg) for an adjustment period of 4 wk. Then, two dogs (−E1 and −E2) were selected randomly to receive the same diet without vitamin E (TD No. 87237, α-tocopherol 0.05 ± 0.02 mg/kg) and two dogs (+E1 and +E2) continued on the supplemented diet. The detailed composition of the diet has been reported elsewhere (13). Diets were ordered in batches of 50 kg and stored at 4°C for a maximum of 8 wk. Samples from each shipment were collected toward the end of the storage period for measurement of α-T to verify that the supplemented diet contained approximately 120 IU vitamin E/kg, the manufacturer's added vitamin E content prior to mixing.

At the end of the 4-wk adjustment period, blood was collected by jugular venipuncture into heparinized 10-mL Vacutainer tubes (143 USP units of heparin; Becton-Dickinson Co., Rutherford, NJ) and immediately placed on ice. The tubes were centrifuged (Model CRU-5000, rotor No. 269; IEC, Needham Heights, MA) at 1500 × *g* for 10 min, and 1-mL aliquots of plasma were removed and stored at −80°C for α-T analysis (see below). The following day, physical and neurologic examinations were

conducted. Then, the dogs were premedicated with acepromazine maleate (0.5 mg/kg body weight, i.m., Atravet; Ayerst Laboratories, Montreal, Quebec, Canada) and atropine (0.05 mg/kg i.m., Fort Dodge Laboratories, Fort Dodge, IA) and anesthetized with Pentothal (18 mg/kg body weight, IV; Abbott Laboratories, North Chicago, IL). Sensory NCV of the superficial radial and peroneal nerves and motor NCV of the ulnar and tibial nerves were determined, as described (14).

Bleeding and electrodiagnostics were repeated at approximately 8-wk intervals for 109 wk. From 24 wk onward, adipose tissue samples were biopsied from subcutaneous fat in the popliteal fossa; samples were wrapped in aluminum foil, immediately frozen on dry ice and stored at −80°C for α-T analysis. At 65 wk, fascicular biopsies of the tibial nerve from the left side (just proximal to the calcaneal tuber) were obtained under anesthesia.

Half of the nerve biopsy specimen was wrapped in foil, frozen on dry ice and stored at −80°C for α-T analysis, as described below. The other half was stretched on a wooden tongue depressor and fixed in 3% glutaraldehyde (4°C) for a week, followed by osmication, dehydration and embedding in epon. Semithin (1 μm) sections of epon embedded nerves were cut, stained with *p*-phenylenediamine and examined by light microscopy (15). Stained sections of nerves were projected onto a monitor, using a computerized video-display image analysis system (Optomax semiautomatic image analysis system; Optomax, Hollis, NH), and mean external diameters of 500 randomly selected fibers were measured (15).

At 109 wk, the dogs were euthanized by an overdose of Pentothal, and 2-cm sections of the tibial nerve from the right side (same location as the biopsy) were collected and processed as described for the biopsies.

α-Tocopherol, triglyceride and cholesterol analyses. Samples of diet, plasma, adipose tissue and nerves were shipped on dry ice, within 7 d of collection, to New York University School of Medicine for analysis of α-T within 1 mon of receipt. Plasma α-T levels were analyzed, as described (16). Adipose tissue α-T, cholesterol and triglycerides (TG) and nerve α-T and cholesterol were analyzed, as described (3,7,17). Data are reported as the mean ± standard deviation (SD).

Semilog plots depicting plasma and adipose tissue α-T concentrations over time were constructed, and linear regression analysis was used to determine depletion rates (Delta Graph Professional, Delta Point, Monterey, CA). Because the decay curves were exponential functions following first-order kinetics, the time to deplete half of the α-T was calculated from the equation: $t_{1/2} = \ln(2)/\beta$, where β is the elimination rate constant (given by the slope) for each curve (18).

RESULTS

Physical and neurologic examination. No abnormalities (rough skin coat, hind limb ataxia or depressed tendon reflexes) were detected in the dogs during physical and neurologic examinations. All dogs maintained their body weights throughout the study (−E1, 18 ± 4 kg; −E2, 19 ± 6; +E1, 18 ± 5; +E2, 19 ± 6).

Plasma and adipose tissue α-T. Baseline plasma α-T concentrations of the four dogs were 25 ± 6 μg/mL (range, 18.4 to 31.3 μg/mL). Throughout the study (14 measurements), plasma α-T values in +E1 were 24 ± 3 μg/mL and in +E2 were 28 ± 5 μg/mL. In −E dogs, semilog plots showed a linear decline in plasma α-T concentrations (Fig. 1); values were <1.0 μg/mL by 65 wk (455 d) and <0.1 μg/mL by 109 wk (763 d). The times to deplete half of the plasma tocopherol ($t_{1/2}$), estimated from the slopes of the plasma α-T concentration curves, were 90 d for −E1 and 85 d for −E2 (Fig. 1).

Shown in Figure 2 are the α-T contents (ng) of adipose tissue expressed per mg wet weight, per mg TG and per μg cholesterol. These three denominators were chosen because (i) wet weight can vary if the specimen becomes dried, (ii) TG have been used as the denominator for measurements of needle biopsies in humans where wet weights are not available and (iii) cholesterol is extracted during α-T isolation and can be conveniently measured. Cholesterol/TG ratios were calculated and found to be relatively constant throughout the study, thereby demonstrating the relative uniformity of the biopsy specimens (−E1, 2.3 ± 1.3 μg/mg; −E2, 1.8 ± 0.6; +E1, 2.4 ± 2.1; +E2, 2.1 ± 1.3).

In +E dogs, adipose tissue α-T concentrations remained high throughout the study (+E1, 450 ± 110 μg/g wet weight; +E2, 320 ± 180) (Fig. 2). Adipose tissue α-T/TG in +E dogs (730 ± 280 μg/mg and 470 ± 250, for +E1 and 2, respectively) are somewhat higher than those obtained by needle biopsies of subcutaneous adipose tissue from nine healthy adult humans (402 ± 154 μg/mg) (19).

Semilog plots of adipose tissue α-T concentrations in −E dogs decreased linearly, and were similar in both −E dogs, whether expressed per wet weight (Fig. 2A), TG (Fig. 2B) or cholesterol (Fig. 2C). The $t_{1/2}$ estimated from Figures 2A, B and C for −E1 were 103, 91 and 104 d and for −E2 were 133, 121 and 165 d; the overall mean was 120 d.

Nerve α-T concentrations, NCV and histopathology. At 65 wk, α-T/cholesterol in tibial nerves of −E dogs were reduced to about 16% of +E values, and by 109 wk, had decreased to about 2% (Table 1).

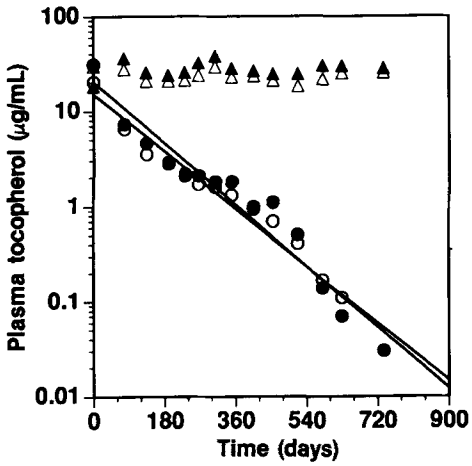

FIG. 1. The semilog plots of plasma α-tocopherol (α-T) in dogs fed a vitamin E-supplemented diet (+E dogs) (△, +E1, ▲, +E2) are relatively constant, while in dogs fed a vitamin E-deficient diet (−E dogs) (○, −E1; ●, −E2) concentrations decline linearly on the semilog plot (−E1, $r^2 = 0.97$; −E2, $r^2 = 0.94$). The half-life of plasma α-T in −E dogs, given by the equation $t_{1/2} = \ln(2)/\beta$ where β is the elimination rate constant of the exponential function (−0.0077 and −0.0082 in −E1 and 2, respectively) is 90 d for −E1 and 85 d for −E2.

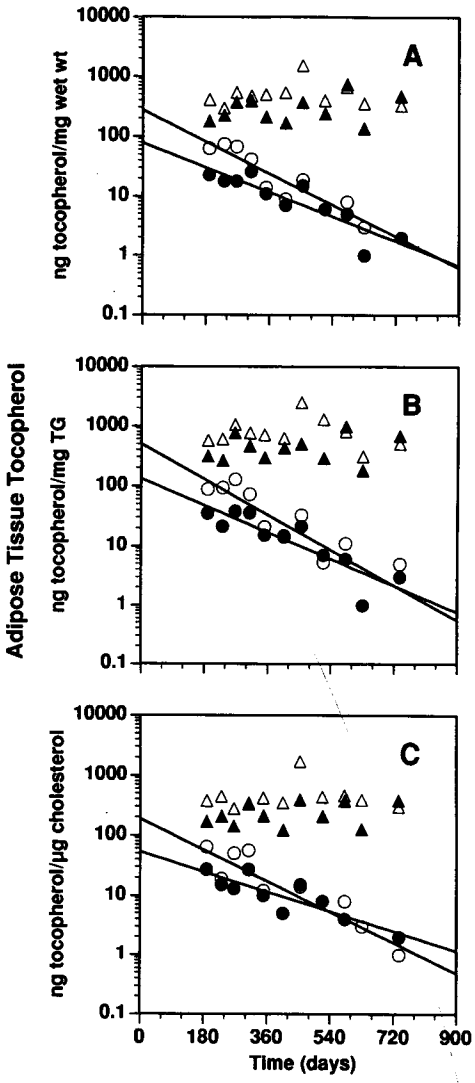

FIG. 2. Semilog plots of adipose tissue α-T concentrations (A) per wet weight, (B) per triglycerides (TG), and (C) per cholesterol are shown. In +E dogs (△, +E1; ▲, +E2), the values remained relatively constant throughout the study. In −E dogs (○, −E1; ●, −E2), the declines in adipose tissue α-T concentrations were linear on a semilog plot for each of the denominators (wet wt, r^2 = 0.90, 0.79; TG, r^2 = 0.77, 0.77; cholesterol, r^2 = 0.80, 0.75, for −E1 and −E2, respectively). The time to deplete half of initial concentrations ($t_{1/2}$) values were calculated as described in Figure 1. The β values were similar for each of the denominators (in −E1, β = −0.0067, −0.0076, −0.0066; in −E2, β = −0.0052, −0.0057, −0.0042; from A, B and C, respectively). The $t_{1/2}$ values calculated from A, B and C for −E1 were 103, 91 and 104 d and for −E2 were 133, 121 and 165 d, respectively. See Figure 1 for other abbreviations.

The sensory and motor NCV values were similar in all dogs throughout the study (Fig. 3). Sensory action potential amplitudes were also measured and were similar in all dogs throughout the study (data not shown).

At 65 and 109 wk, no detectable differences between −E and +E nerves were apparent by light microscopy. Electron microscopic examination of semithin sections of the tibial nerve did not reveal any abnormalities (such as loss of large-diameter myelinated fibers) at either 65 or 109 wk. By 109 wk, mean external diameters of myelinated nerve fibers were not different between the two groups (Table 1).

TABLE 1

α-T/Cholesterol Ratios and Diameters of Tibial Nerves from Dogs Fed Vitamin E-Deficient (−E) or Supplemented (+E) Diets

Time (wk)	Dog	Nerve composition (μg α-T/mg cholesterol)	Nerve diameter (μm ± SD)
65	+E1	6.3	na[a]
	+E2	5.6	na
	−E1	0.4	na
	−E2	1.5	na
109	+E1	3.6	5.1 ± 2.6
	+E2	6.7	4.1 ± 2.0
	−E1	0.04	3.8 ± 1.6
	−E2	0.09	4.8 ± 2.6

[a]na, Not analyzed; abbreviations: α-T, α-tocopherol; SD, standard deviation.

DISCUSSION

This is the first longitudinal study measuring plasma and adipose tissue α-T concentrations in dogs fed vitamin E-supplemented or -deficient diets over 109 wk. In −E dogs, both plasma and adipose tissue α-T concentrations decreased with first-order kinetics. Estimated values of the $t_{1/2}$ for depletion of α-T from plasma were 87 d and from adipose tissue were 120 d. These data suggest that dog adipose tissue α-T stores are utilized for the maintenance of plasma α-T. Plasma and adipose tissue α-T concentrations in −E dogs were correlated (−E1, r^2 = 0.7942, $P < 0.0003$; −E2, r^2 = 0.7930, $P < 0.0003$), giving further support to this hypothesis.

Plasma α-T concentrations also decrease with first-order kinetics in response to a vitamin E-deficient diet in other species, such as rats (6,20), guinea pigs (5), monkeys (6) and humans (for a review, see 21). Adipose tissue α-T also decreases with first-order kinetics in rodents. We have estimated, using previously reported data, that the $t_{1/2}$ of α-T in adipose tissue of mature rats was 70 d (20), and of mature guinea pigs was 600 d (5). Thus, the $t_{1/2}$ of α-T in adipose tissue is longer in the dog compared to the rat, but much shorter than the guinea pig.

By 65 wk, −E dogs had tibial nerve concentrations (Table 1), which were comparable to those from sural nerves of vitamin E-deficient humans (1.8 ± 1.2 ng/μg cholesterol) (3). By 109 wk, α-T concentrations of tibial nerves in −E dogs were well below deficient human sural nerve levels. By contrast, weanling rats fed vitamin E-deficient diets had a faster decline of α-T from nerve (4–7% of control sciatic-tibial nerve values by 36 wk) compared to −E dogs (16% of control tibial nerve values by 65 wk). Furthermore, vitamin E-deficient rats had decreased tibial NCV by 8 mon (22) and axonal swelling in sural nerves by 12 mon (23). The earlier onset of changes in rats may be attributed to the younger age when vitamin E-deficient diets were initiated and to species differences.

The −E dogs did not develop neurologic abnormalities, and electrophysiologic tests remained normal (Table 1). The lack of slowing in nerve conduction velocities in our study is similar to findings in vitamin E-deficient humans with subclinical disease (24). In nine patients with serum

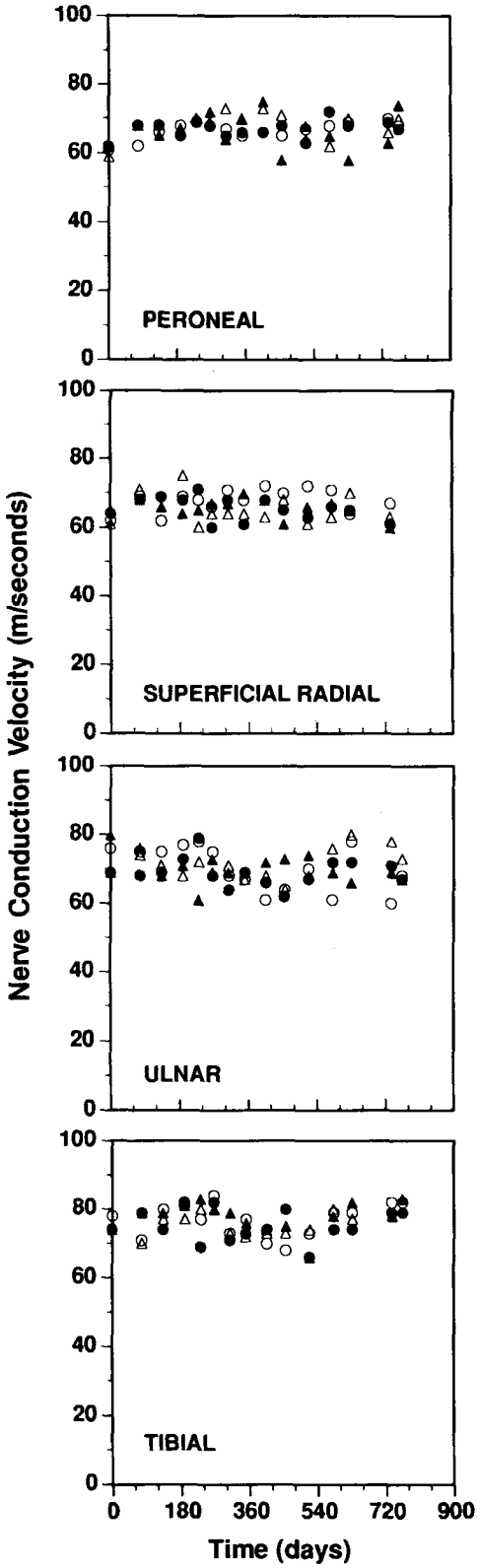

FIG. 3. Sensory nerve conduction velocity (NCV) of the peroneal and superficial radial nerves, and motor NCV of the ulnar and tibial nerves in dogs fed vitamin E-deficient (○, −E1; ●, −E2) or control diets (△, +E1; ▲, +E2) were similar in both groups at all times.

α-T < 5 μg/mL, three were clinically asymptomatic and did not show any electrophysiologic abnormalities. The remaining six patients had varying degrees of neurologic abnormalities including weakness, proprioceptive deficits and ataxia, and had delayed central conduction measured by somatosensory evoked potentials (24). These findings and a previous report in humans (3) suggest that nerves must be depleted of α-T for years before histologic changes occur.

In conclusion, plasma α-T in adult dogs fed vitamin E-deficient diets declines in parallel with adipose tissue α-T. The correlation of plasma α-T with adipose tissue α-T suggests that the adipose tissue α-T can be taken up by plasma lipoproteins for delivery to other tissues in the body (as reviewed in Ref. 25). The development of vitamin E deficiency in adult dogs resembles that in humans more closely than in rodents in terms of tissue α-T depletion, and the relatively prolonged duration of the deficient state before development of clinical, electrophysiologic and/or histopathologic signs.

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α-Tocopherol Concentrations of the Nervous System and Selected Tissues of Adult Dogs Fed Three Levels of Vitamin E

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The effects of dietary vitamin E levels on tissue α-tocopherol (α-T) concentrations in different parts of the nervous system are largely unknown. Therefore, we measured the α-T contents of nervous and other tissues obtained from beagle dogs fed for two years a vitamin E-deficient diet (−E, 0.05 ± 0.02 mg vitamin E/kg diet, n = 2), a vitamin E-supplemented diet (+E, 114 ± 14 mg/kg, n = 2), or a standard chow diet (E_n, 74 ± 6 mg/kg, n = 3). Brain regions and spinal cords of +E dogs contained about double the α-T concentrations of E_n dogs, and about 10-fold those of −E dogs. The various brain regions of −E dogs, compared with E_n dogs, retained 12-18% of the α-T concentrations, with the exception of the caudal colliculus, which retained 48%. Peripheral nerve α-T concentrations in +E dogs (67 ng/mg wet weight) were nearly 5-fold higher than in E_n dogs (13.4 ± 5.9 ng/mg) and 80-fold higher than in −E dogs (0.8 ng/mg). Within each dietary group, the lowest α-T concentrations in the central nervous system (CNS) were in the spinal cord. Peripheral nerves were the most susceptible to vitamin E repletion or depletion: in +E dogs, nerves contained higher concentrations of α-T than most brain regions; in E_n dogs, they contained similar concentrations; but in −E dogs, they contained less α-T than most brain regions. Muscles and other tissues of −E dogs retained from 1 to 10% of E_n values. The studies demonstrate that the CNS conserved α-T compared to peripheral nerves and nonnervous tissues in adult dogs, but contained lower absolute concentrations of α-T compare with most other tissues.

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Vitamin E deficiency symptoms in humans and in experimental animals include a progressive peripheral neuropathy (1). Our hypothesis is that the development of pathological changes resulting from vitamin E deficiency depends upon the tissue concentrations of α-tocopherol (α-T), which reflect the storage capacity of the tissue (*i.e.*, the lipid content of the tissue), α-T utilization rates and α-T replacement rates. For example, in rodents the nervous system conserves α-T compared to liver, heart and muscle (2,3). However, the cerebellum of rodents is particularly susceptible to vitamin E deficiency, presumably because the low α-T concentrations reflect high utilization despite a high uptake of α-T, as measured with radioactive α-T (4).

To investigate the effects of vitamin E deficiency on nervous tissue concentrations of α-T in adult dogs, four male 1-year-old beagle dogs were evaluated; two were fed a vitamin E-deficient diet (−E), and two were fed the same diet supplemented with vitamin E (+E) for two years. Additionally, tissues from three female 5-year-old beagles that were fed a standard chow diet were analyzed. Concentrations of α-T in the central nervous system (CNS), peripheral nerves, skeletal muscles and selected tissues were determined.

MATERIALS AND METHODS

Protocol. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee (Auburn, AL). As described in other works (5,6), four 1-year-old male beagle dogs (Marshall Farms, North Rose, NY), weighing about 18 kg, were fed a purified diet containing *all-rac*-α-tocopheryl acetate (TD No. 87238; Teklad Laboratories, Madison, WI; α-T, 114 ± 14 mg/kg) for an adjustment period of 4 wk. Then, two dogs (−E1 and −E2) were selected randomly to receive the same diet without vitamin E (TD No. 87237, α-T 0.05 ± 0.02 mg/kg), and two dogs (+E1 and +E2) continued on the supplemented diet.

The dogs (3-years-old), following maintenance on the diets for two years, were euthanized by an overdose of intravenous barbiturate (Pentothal; Abbott Lab; North Chicago, IL); tissues were collected for measurement of α-T as described below. Additional tissues from three healthy female 5-year-old beagles maintained for two years in the same facility were collected. These dogs (designated E_n) had received a standard laboratory diet (Dog Chow; Purina Mills, St. Louis, MO) containing added *all-rac*-α-tocopheryl acetate (measured α-T content, 74 ± 6 mg/kg).

Tissue collection. Immediately after euthanasia, the cranium was opened and the entire brain was removed and placed on ice. The left half was used to dissect out 500-mg pieces of frontal, parietal, temporal and occipital cortex, caudate nucleus, hippocampus, amygdala, rostral and caudal colliculus, cerebellum, pons and medulla. The spinal cord was dissected into cervical (C 1), thoracic (T 6-7) and lumbar (L 5-6) segments, and 500-mg pieces from the dorsal columns were collected. Samples (1 cm) of the sciatic (at the coxo-femoral joint), tibial (proximal to the tarsus) and superficial radial (at the elbow joint) nerves, and the rectus femoris and extensor carpi radialis muscles were excised. Pieces (1 g) of liver, cardiac muscle (left ventricle), adrenal gland, renal cortex and medulla, and testicles were collected also. All tissue samples were stripped of connective tissue and fat, immediately wrapped in aluminum foil and frozen on dry ice. The samples were stored at −80°C until shipment, within 1 wk of collection, to New York University School of Medicine for α-T analysis. The α-T and cholesterol contents of all samples were analyzed, as previously reported (7,8), within two months of receipt.

Statistical analysis. Average α-T concentrations of tissues for +E and −E dogs, and means ± standard deviations for E_n dogs were calculated [Super ANOVA; Abacus Concepts, Berkeley, CA). For E_n dogs, one-way analysis of variance and comparisons of least square means were used to determine differences in α-T concentrations in the brain and spinal cord samples, and peripheral nerves. A value of $P < 0.05$ was considered significant.

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Abbreviations: α-T, α-tocopherol; ANOVA, analysis of variance; CNS, central nervous system; +E, dogs fed vitamin E-supplemented diet; −E, dogs fed vitamin E-deficient diet; E_n, dogs fed standard chow diet; SD, standard deviation.

RESULTS

Brain regions. The α-T contents of various anatomical sites from the brains of the three groups of dogs fed different dietary levels of vitamin E are shown in Table 1. Because α-T concentrations in specific neuroanatomic areas of the brain did not differ markedly, brain regions were grouped according to embryological divisions, *viz.*, telencephalon (frontal, parietal, occipital and temporal cortices, caudate nucleus, hippocampus and amygdala), diencephalon (thalamus and hypothalamus), mesencephalon (rostral and caudal colliculus), metencephalon (cerebellum and pons) and myelencephalon (medulla oblongata). Mean α-T concentrations for these regions and the statistical significance between regions in E_n dogs are also shown in Table 1.

In E_n dogs, α-T concentrations in the telencephalon were significantly greater than those of the medulla oblongata. Furthermore, the rostral brain regions (telencephalon, diencephalon), irrespective of diet group, contained higher α-T concentrations than the caudal areas (mesencephalon, metencephalon, myelencephalon).

On an α-T/cholesterol basis, the telencephalon in E_n dogs contained significantly greater concentrations ($P < 0.05$) than the other brain regions. Indeed, the α-T/cholesterol ratios were markedly higher in the telencephalon compared with the myelencephalon in all dogs. The α-T/cholesterol ratios were high in the telencephalon because this region contained the lowest cholesterol concentrations, while the myelencephalon contained the highest. It should be noted that the cholesterol concentrations of each of the brain regions were similar between the three dietary groups (Table 1).

In −E dogs, α-T concentrations of the brain regions were minimal, ranging from 0.7 to 3.8 ng/mg wet wt (Table 1), about 12–18% of those of E_n dogs, with the exception of the mesencephalon (Fig. 1). In the mesencephalon, the caudal colliculus of −E dogs contained 48% of α-T concentrations in E_n dogs. However, the caudal colliculus in −E dogs did not contain high concentrations of α-T, nor was it particularly enriched with α-T in either +E or E_n dogs (Table 1).

Spinal cord. The spinal cord α-T/wet weight ratios were significantly lower than in the telencephalon and the peripheral nerve in E_n dogs (Table 1). Again the high cholesterol content of the spinal cord caused the α-T/cholesterol ratio to be one of the lowest in the nervous system. In E_n dogs, the spinal cord α-T/cholesterol ration was 1/8 of that of the telencephalon ($P < 0.05$).

Spinal cord α-T contents (expressed per weight or per cholesterol) in +E dogs were double those in E_n dogs, and more than 10-fold those in −E dogs (Table 1).

Peripheral nerves. Of all nervous tissues, peripheral nerves were the most susceptible to vitamin E repletion or depletion (Table 1 and Fig. 1). In +E dogs, nerve α-T concentrations were more than double those of most brain regions, or spinal cord. In E_n dogs, nerve α-T concentrations were significantly greater than in the mesencephalon, metencephalon, myelencephalon or the spinal cord. However, in −E dogs, nerve α-T concentrations were about half of most other brain regions. Peripheral nerves of −E dogs contained about 6% of those of E_n nerves, the lowest percentage retained of all the nervous tissues (Fig. 1), but comparable to α-T retention in nonnervous tissues (see below).

TABLE 1

α-Tocopherol (α-T) Concentrations of Various Neuroanatomic Regions of Brain, Spinal Cord and Peripheral Nerve from Dogs Fed Vitamin E-Deficient (−E1, −E2), -Supplemented (+E1, +E2) or Control (E_n, n = 3) Diets

Region	ng α-T/mg wet wt					ng α-T/μg cholesterol					μg Cholesterol/mg wet wt				
	−E1	−E2	+E1	+E2	$E_n \pm SD$	−E1	−E2	+E1	+E2	$E_n \pm SD$	−E1	−E2	+E1	+E2	$E_n \pm SD$
Frontal cortex	2.4	2.0	36	30	14 ± 8	0.18	0.17	2.0	1.8	0.5 ± 0.5	13	11	18	16	19 ± 5
Parietal cortex	1.6	0.8	24	33	12 ± 1	0.11	0.04	1.3	2.3	1.1 ± 0.1	14	19	18	14	11 ± 2
Occipital cortex	1.8	1.6	33	36	13 ± 3	0.10	0.09	2.8	2.0	0.5 ± 0.3	17	19	12	18	21 ± 4
Temporal cortex	1.7	1.5	27	26	11 ± 2	0.12	0.10	2.8	2.5	0.9 ± 0.2	14	15	10	10	11 ± 1
Caudate nucleus	1.5	2.0	22	31	10 ± 3	0.14	0.16	1.8	2.7	0.5 ± 0.3	11	12	12	11	18 ± 5
Hippocampus	0.9	1.7	33	12	9 ± 4	0.04	0.08	1.8	0.4	0.7 ± 0.4	24	22	18	30	14 ± 8
Amygdala	1.0	1.5	8	29	8 ± 1	0.07	0.14	0.5	2.5	0.7 ± 0.2	14	11	17	12	13 ± 2
Telencephalon (mean)	1.5	1.6	26	28	11 ± 4[a,b,c]	0.11	0.11	1.9	2.0	0.8 ± 0.3[a–e]	15	16	15	16	16 ± 5[a–d]
Thalamus	2.4	1.9	36	14	15 ± 7	0.10	0.10	1.4	0.5	0.6 ± 0.3	24	20	26	26	22 ± 2
Hypothalamus	0.3	0.9	17	23	5 ± 1	0.02	0.06	0.8	1.1	0.2 ± 0.1	22	15	21	22	23 ± 1
Diencephalon (mean)	1.4	1.4	27	18	10 ± 7[d]	0.06	0.08	1.1	0.8	0.5 ± 0.4[a,f]	23	18	24	24	23 ± 2[e]
Rostral colliculus	1.6	1.6	15	42	7 ± 3	0.04	0.06	0.4	1.3	0.3 ± 0.2	37	27	36	33	40 ± 16
Caudal colliculus	2.3	2.2	8	17	5 ± 3	0.09	0.10	0.5	0.7	0.2 ± 0.1	25	21	18	25	27 ± 10
Mesencephalon (mean)	1.9	1.9	12	29	6 ± 3[a,e]	0.07	0.08	0.4	1.0	0.2 ± 0.1[b,g]	31	24	27	29	30 ± 14[a,f,g]
Cerebellum	0.7	0.6	23	23	8 ± 1	0.05	0.03	1.4	1.7	0.4 ± 0.18	16	19	16	14	17 ± 3
Pons	0.4	2.4	12	15	9 ± 1	0.01	0.07	0.2	0.3	0.2 ± 0.02	34	37	52	49	41 ± 4
Metencephalon (mean)	0.5	1.5	17	19	8 ± 1[f]	0.03	0.05	0.8	1.0	0.4 ± 0.20[c,h]	25	28	34	31	30 ± 16[b,h,i]
Myelencephalon	0.0	1.3	2	12	4 ± 3[b,d,g]	0.00	0.03	0.1	0.2	0.2 ± 0.1[d,i]	22	49	24	53	33 ± 17[c,j,k]
Cervical cord	1.8	2.6	59	22	6 ± 1	0.03	0.05	1.0	0.4	0.1 ± 0.02	52	58	57	56	54 ± 2
Thoracic cord	0.6	1.5	14	17	7 ± 2	0.01	0.03	0.2	0.2	0.1 ± 0.04	62	57	60	68	59 ± 4
Lumbar cord	3.8	0.2	17	18	7 ± 3	0.08	0.00	0.4	0.4	0.2 ± 0.07	45	54	46	46	48 ± 3
Spinal cord (mean)	2.0	1.4	30	19	7 ± 2[c,h]	0.04	0.02	0.5	0.3	0.1 ± 0.04[e,j]	53	56	55	57	53 ± 5[d–f,h,j,l]
Superficial radial nerve	1.5	0.6	60	83	18 ± 5	0.24	0.08	9.5	14.8	1.4 ± 0.4	7	8	6	6	12 ± 1
Sciatic nerve	0.6	0.8	56	64	11 ± 1	0.03	0.04	3.9	4.5	0.6 ± 0.1	18	17	14	14	17 ± 5
Tibial nerve	0.8	0.8	60	80	11 ± 8	0.04	0.09	3.6	6.7	0.9 ± 0.6	19	9	17	12	13 ± 1
Peripheral nerve (mean)	1.0	0.7	58	76	13 ± 6[e–h]	0.10	0.07	5.6	8.7	1.0 ± 0.6[f–j]	14	11	12	11	15 ± 5[g,i,k,l]

[a–l]Regions of E_n dogs in each column bearing the same superscript letter are significantly different ($P < 0.05$) by least squares means comparisons.

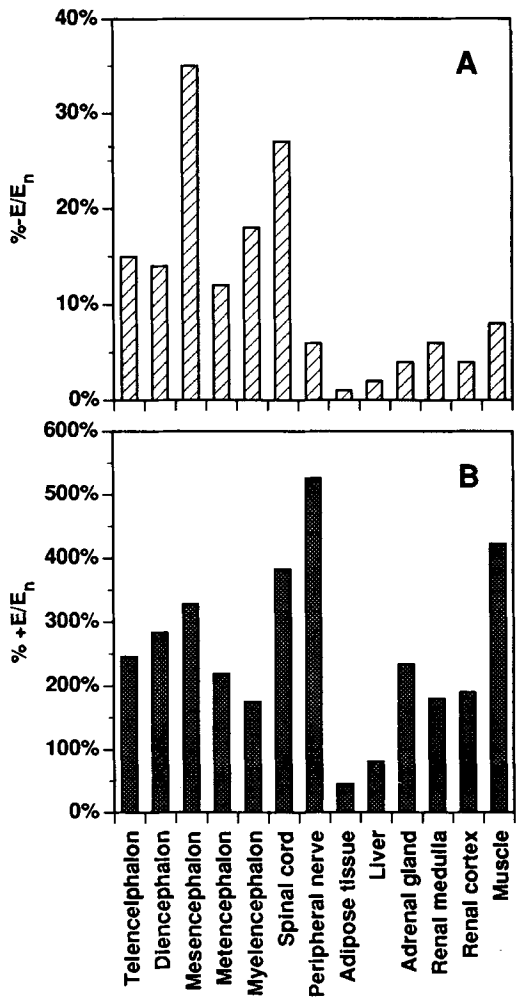

FIG. 1. A. α-Tocopherol (α-T) concentrations of tissues from two dogs fed vitamin E-deficient diets (−E), expressed as a % of mean E_n dog α-T concentrations, *i.e.*, % = $-E/E_n \times 100$. B. α-T concentrations of tissue from two dogs fed vitamin E-supplemented diets (+E), expressed as a % of E_n dog α-T concentrations, *i.e.*, % = $+E/E_n \times 100$.

Because of their relatively low cholesterol contents compared with other parts of the CNS (except telencephalon and diencephalon), α-T/cholesterol ratios were significantly ($P < 0.05$) higher in E_n peripheral nerves than in mesencephalon, metencephalon, myelencephalon and spinal cord.

Other tissues. α-T concentrations were followed longitudinally in −E and +E dogs, as reported in the preceding paper (6). In +E dogs, adipose tissue α-T concentrations remained high throughout the study [450 ± 110 μg/g wet weight and 320 ± 180 for +E 1 and 2, respectively (6)], and were similar to those in E_n dogs (376 μg/g) (Table 2).

In −E dogs, α-T was depleted from adipose tissue with first order kinetics to 1.6 ng/mg wet wt in −E1 and 1.9 in −E2 (6). However, 1 wk prior to sacrifice, these dogs were given a dose of deuterated α-tocopherol [4 mg/kg body weight each of *RRR-* and *all rac-α*-tocopheryl acetates, 142 mg total (9)], which apparently raised the adipose tissue values to 7.9 ng/mg wet weight in −E1 and 2.6 in −E2, as shown in Table 2.

The liver contained similarly high α-T concentrations in both +E dogs and in E_n dogs (Table 2). Thus, the liver did not store additional α-T in response to supplementation. The liver in −E dogs contained 3.2 ng/mg, only 1.6% of that in E_n liver (Fig. 1).

High α-T concentrations were found in the adrenal glands of both +E and E_n dogs, but were depleted in −E dogs to 3.5% of E_n values (Table 2 and Fig. 1). The high cholesterol contents of the adrenal glands (20–30 μg/mg wet weight) decreased the ratios of α-T/cholesterol to values lower than those in most other nonnervous tissues (Table 2).

Irrespective of diet, the renal medulla contained 8–10 times more α-T than did the cortex on a wet weight basis and 4–5 times more on a cholesterol basis (Table 2). The higher α-T contents of the medulla can be explained by differences in lipid contents. On histologic examination of fixed tissue, the medulla was observed to have more lipid than the cortex. The cholesterol contents confirm this observation; the medulla contained roughly double the cholesterol concentration of the cortex.

Within each dietary group, α-T concentrations (either per wet weight or per cholesterol) of the three different muscles (cardiac and the two skeletal muscles) were similar (Table 2). The average α-T concentration of muscles from +E dogs were approximately 4-fold greater than those of E_n and 50-fold greater than those of −E dogs.

Testicles of +E dogs contained about 50 times more α-T than those of −E dogs (Table 2).

DISCUSSION

The distribution of α-T in brain regions of dogs fed three dietary levels of vitamin E was measured in an effort to

TABLE 2

α-Tocopherol (α-T) Concentrations of Nonnervous Tissues from Dogs Fed Vitamin E-Deficient (−E1, −E2), -Supplemented (+E1, +E2) or Control (E_n, n = 3) Diets

		ng α-T/mg wet wt					ng α-T/μg Cholesterol					μg Cholesterol/mg wet wt				
Region		−E1	−E2	+E1	+E2	E_n ± SD	−E1	−E2	+E1	+E2	E_n ± SD	−E1	−E2	+E1	+E2	E_n ± SD
Adipose tissue	Popliteal fossa	7.9	2.6	110	230	406, 346[a]	11.1	2.6	115	324	509, 403[a]	0.7	1.0	1.0	0.7	0.8, 0.9[a]
Liver		4.0	2.4	131	185	194 ± 55	1.4	0.9	48	56	54 ± 26	2.9	2.5	2.7	3.3	3.5 ± 0.6
Adrenal gland		5.9	3.1	277	336	131 ± 50	0.4	0.2	19	12	4 ± 1	16	21	14	27	28 ± 8
Kidney	Renal medulla	3.9	8.2	237	148	107 ± 31	1.1	2.3	55	45	22 ± 7	3.4	3.6	4.3	3.3	4.6 ± 0.5
	Renal cortex	0.7	0.5	24	25	13 ± 6	0.3	0.2	14	13	4 ± 2	1.9	2.8	1.8	2.0	2.4 ± 0.3
Muscle	Cardiac	0.9	0.7	65	31	11 ± 2	0.5	0.6	40	30	8 ± 1	1.7	1.2	1.6	1.0	1.3 ± 0.2
	Extensor carpi radialis	0.9	0.7	29	25	9 ± 2	1.0	1.0	31	50	10 ± 2	0.9	0.7	0.9	0.5	0.8 ± 0.2
	Rectus femoris	0.7	0.8	63	43	10 ± 5	0.9	2.3	83	58	15 ± 3	0.8	0.4	0.8	0.7	0.6 ± 0.3
Testicle		0.5	1.2	55	62	—[b]	0.5	0.3	30	32	—[b]	1.2	3.4	1.9	1.9	—[b]

[a]Adipose tissue was obtained from only two dogs.
[b]Not available.

assess which brain regions are responsive to changes in dietary α-T. In response to vitamin E deficiency, the lowest concentrations of α-T in −E dogs were found in the cerebellum (0.7 ng/mg) and the myelencephalon (medulla oblongata) (0.7 ng/mg); the cerebellum retained the lowest percentage of α-T (9%). Previous studies in rats (2,3) and mice (10) have shown that the cerebellum is readily depleted of α-T, and is an active site of uptake of radioactive vitamin E. Typically, axonal dystrophy occurs in the cerebellum and brainstem in experimental vitamin E deficiency in rodents and monkeys (11), and immature dogs (12). Dystrophic changes were also seen in the brainstem nuclei of −E dogs (Pillai, S.R., and Steiss, J.E., unpublished observations).

In −E dogs, the brain region which retained the largest percentage of α-T (48%) was the caudal colliculus, part of the auditory pathway. Apparently, the auditory pathway is more resistant to vitamin E depletion than the visual and somatosensory tracts. Consistent with our study are findings in vitamin E-deficient humans. No changes in brainstem auditory-evoked potentials were reported, although these patients had delayed somatosensory-evoked potentials, abnormal electroretinograms and delayed visual-evoked potentials (13). Similarly, in vitamin E-deficient rats, brainstem auditory-evoked potentials are normal (14), while somatosensory-evoked potentials (14,15), electroretinograms and visual-evoked potentials (14) are abnormal.

Brain regions of dogs and rodents appear to respond somewhat differently to vitamin E supplementation. In +E dogs, the highest increase of α-T occurred in the mesencephalon, while in vitamin E-supplemented rats, maximum increases have been reported in the medulla oblongata and pons (2), and the cerebellum (16). By contrast, the medulla in +E dogs was the brain region with the smallest increase in α-T concentrations (Table 1).

Peripheral nerves were remarkable in having the highest α-T concentration of all nervous tissue in +E dogs, as previously observed in rats (4,17). Furthermore, in +E dogs, the increases in nerve α-T concentrations were about double those of the CNS. Correspondingly, in −E dogs, the nerves retained lower percentages (6%) of α-T than other portions of the nervous system. The ease with which peripheral nerves are depleted may explain why humans (18), monkeys (11) and rats (19) develop a peripheral neuropathy associated with vitamin E deficiency. However, in the present study, −E dogs did not develop clinical, electrophysiological or histologic evidence of peripheral neuropathy (Pillai, S.R., and Steiss, J.E., unpublished data).

Of other tissues examined, the highest α-T concentrations in response to vitamin E supplementation were in adipose tissue, liver, adrenal gland and kidney (Table 2). Adipose tissue is a major α-T storage site in humans (7), rats (20), guinea pigs (21), mink (22) and horses (23). The liver also accumulates α-T with vitamin E supplementation in most species (20–25). The accumulation of α-T by the adrenal gland in +E dogs is in agreement with studies in humans (26), rats (27) and cattle (28,29). These high α-T concentrations may be related to the high cholesterol contents of the adrenal gland, or higher antioxidant levels may be required to protect the tissue during steroid hormone synthesis (26). We found higher α-T concentrations in the renal medulla compared with the cortex, which are likely due to the higher lipid contents of the medulla.

In dogs, irrespective of diet, cardiac and skeletal muscle contained similar α-T concentrations, but in pigs (25), cattle (28) and sheep (30), cardiac muscles contained higher α-T concentrations than skeletal muscles. These may represent species differences.

In conclusion, the present study demonstrates in dogs that of all nervous tissues, peripheral nerves were most responsive to vitamin E supplementation and deficiency. In −E dogs, the CNS retained higher percentages of α-T than either peripheral nerves or non-nervous tissues; however, the absolute α-T concentrations in nervous tissues were lower than in most other tissues. Considering vitamin E functions as an antioxidant, and that tissues depleted of α-T are susceptible to free radical damage, it is therefore not surprising that vitamin E-deficient dogs display neuromuscular abnormalities (12,31).

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Vitamin E Deficiency in Dogs Does Not Alter Preferential Incorporation of *RRR*-α-Tocopherol Compared with *all rac*-α-Tocopherol into Plasma

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The plasma and lipoprotein transport of *RRR* and *all rac*-α-tocopherols, labeled with different amounts of deuterium [2*R*,4′*R*,8′*R*-α-[5-C^2H_3]tocopheryl acetate (*d_3RRR*-α-tocopheryl acetate] and 2*RS*, 4′*RS*, 8′*RS*-α-[5,7-$(C^2H_3)_2$]tocopheryl acetate (*$d_6$all rac*-α-tocopheryl acetate), was studied in adult beagle dogs that had been fed a vitamin E-deficient (−E; two dogs) or supplemented (+E; two dogs) diet for two years. We set out to test the hypothesis that the activity of the hepatic tocopherol binding protein (which is thought to preferentially incorporate *RRR*-α-tocopherol into the plasma) is up-regulated by vitamin E deficiency. Labeled α-tocopherols increased and decreased similarly in plasma of both −E and +E dogs. Irrespective of diet, *d_3RRR*-α-tocopherol was preferentially secreted in plasma. Thus, vitamin E deficiency in dogs does not markedly increase the apparent function of the hepatic tocopherol binding protein. We also studied vitamin E transport in a German Shepherd dog with degenerative myelopathy (DM). Based on the coincident appearance of *d_3RRR*-α-tocopherol in plasma and chylomicrons, we suggest that the abnormality in DM may be associated with abnormal vitamin E transport resulting from an impaired function of the hepatic tocopherol binding protein.
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Vitamin E, a fat-soluble vitamin, is transported in the plasma lipoproteins (for reviews, see Refs. 1 and 2). Vitamin E is absorbed by the intestine and secreted in chylomicrons (3), which are catabolized in the circulation. Chylomicron remnants are then taken up by the liver. Of the various forms of dietary vitamin E, *RRR*-α-tocopherol is preferentially secreted by the liver into the plasma in very low density lipoproteins (VLDL) (3–6). This phenomenon was demonstrated directly using perfused monkey livers from animals fed deuterium-labeled tocopherols (5). The nascent VLDL secreted by the livers were preferentially enriched with *RRR*-, as compared with *SRR*-α-tocopherol or γ-tocopherol (5).

Catabolism of circulating VLDL maintains plasma *RRR*-α-tocopherol concentrations, as shown in studies using deuterated stereoisomers of α-tocopherol (*RRR*- and *SRR*-) or naturally occurring, deuterated forms of vitamin E (α- and γ-tocopherols) in healthy subjects (4), patients with familial, isolated vitamin E deficiency (7,8) and patients with genetic abnormalities of lipoprotein metabolism (6,9). Further, we have proposed that incorporation of *RRR*-α-tocopherol in nascent VLDL requires the action of the hepatic tocopherol binding protein. Such a protein has been purified from rat liver (10,11) and shown to transfer α-tocopherol (in preference to other tocopherols) between liposomes and microsomes (10).

Because of its putative role in maintaining plasma vitamin E levels (2), the tocopherol binding protein or its activity might be increased in response to vitamin E deficiency. However, there are conflicting reports in the literature as to whether its activity is increased (12) or unchanged (11) in response to vitamin E deficiency in rats. In addition, because this protein likely differentiates between forms of vitamin E, secretion of *RRR*-α-tocopherol into the plasma might be enhanced by vitamin E deficiency. Therefore, to test the hypothesis that the activity of the tocopherol binding protein is up-regulated by vitamin E deficiency, we studied the transport of *RRR*- and *all rac*-α-tocopherol labeled with different amounts of deuterium in adult beagle dogs that had been fed a vitamin E-deficient or vitamin E-supplemented diet for two years (−E, +E, respectively).

We also studied vitamin E transport in a German Shepherd dog diagnosed to have degenerative myelopathy (DM) (13). This disease (13–15) has several similarities to vitamin E deficiency in other species (16,17), namely, degenerative changes in the spinal cord, pelvic limb ataxia and paresis, hyporeflexia or areflexia, impaired cellular immunity, and low serum vitamin E levels. Furthermore, a preliminary report suggests that progression of the disease is slowed by the administration of large doses of vitamin E to affected dogs (14). Familial isolated vitamin E deficiency in humans is an inherited disease (18), and DM in dogs primarily affects the German Shepherd breed, suggesting a genetic predisposition. Based on the possibility that DM might be the result of an impaired transport of vitamin E due to a defective hepatic tocopherol binding protein, we tested the ability of this dog to transport deuterated α-tocopherols.

MATERIALS AND METHODS

Animals. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee (Auburn, AL). As described previously (19,20), four one-year-old male beagle dogs (Marshall Farms, North Rose, NY), weighing about 18 kg each, were fed a purified diet containing *all rac*-α-tocopheryl acetate (TD No. 87238; Teklad Laboratories, Madison, WI; α-tocopherol, 114 ± 14 mg/kg) for an adjustment period of 4 wk. Then, two dogs (−E1 and −E2) were selected randomly to receive the same diet without vitamin E (TD No. 87237, α-tocopherol 0.05 ± 0.02 mg/kg) and two dogs (+E1 and +E2) continued on the supplemented diet. The dogs were maintained on the diets for two years. At the time of this study, −E dogs were clinically healthy, but their plasma, red blood cells and tissues were depleted of vitamin E (19–21).

The dog diagnosed to have DM was a nine-year-old male, purebred German Shepherd. His diet consisted primarily of commercial dog food. Ataxia and paresis involving

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Abbreviations: c_{max}, Maximum observed concentrations; DM, degenerative myelopathy; −E, dogs fed a vitamin E-deficient diet; +E, dogs fed a vitamin E-supplemented diet; HDL, high density lipoproteins; LDL, low density lipoproteins; t_{moc}, time of maximum observed concentration; VLDL, very low density lipoproteins.

the pelvic limbs were first noted in March 1991. Three months later, a subcutaneous adipose tissue biopsy from the popliteal fossa and a plasma sample were collected, and the deuterated tocopherol study was performed. At this time, the dog had patellar hyperreflexia, a decreased lymphocyte blastogenesis test to three test antigens, normal findings on cerebrospinal fluid analysis, negative tiers for canine distemper virus (<1:2 for IgG and IgM) and no evidence of a compressive lesion on myelogram. Six months later, the dog was re-admitted for peripheral nerve biopsy for vitamin E analysis. At that time, the dog was paraplegic, and the patellar reflexes were depressed bilaterally. The dog was not receiving any vitamin E supplementation during the time of this study.

Deuterated tocopherols. Deuterated forms of vitamin E [2*R*,4′*R*,8′*R*-α-[5-C^2H_3]tocopheryl acetate (d_3*RRR*-α-tocopheryl acetate] and 2*RS*, 4′*RS*, 8′*RS*-α-[5,7-$(C^2H_3)_2$]tocopheryl acetate (d_6*all rac*-α-tocopheryl acetate) were synthesized by Eastman Chemical Products, Inc. (Kingsport, TN) and were a generous gift from Eastman Chemical Products and the Natural Source Vitamin E Association (Washington, D.C.). Tocopherols were analyzed by gas chromatography/mass spectrometry after conversion to their trimethylsilyl ethers with quantitation by comparison to an added internal standard, 2-*ambo*-α-[5,7,8-$(C^2H_3)_3$]tocopherol, as described previously (3,8,22-24).

Experimental protocols. Following an overnight fast, +E and −E dogs consumed a dose containing 71 mg each of d_3*RRR*- and d_6*all rac*-α-tocopheryl acetates (4.0 mg/kg body weight) dissolved in 1 mL vegetable oil (94 ng unlabeled α-tocopherol/mL and 396 ng γ-tocopherol/mL), and then were given their daily ration of food. Due to a calculation error, the DM dog was given a smaller dose, 63 mg of each isotope (2.1 mg/kg body weight) in 1 mL vegetable oil. Blood samples were obtained at 0, 3, 6, 9, 12, 24, 36, 48 and 72 h. Lipoproteins were isolated from the plasma, as described previously (4), at densities used for human lipoproteins [VLDL, d < 1.006 g/mL; low density lipoprotein (LDL), 1.006 < d < 1.063; and high density lipoprotein (HDL) d > 1.063]. These densities have been used previously to characterize dog plasma lipoproteins (25). The areas under the curves for the plasma and the lipoproteins were calculated using Kaleida Graph (Synergy Software, Reading, PA).

RESULTS

Plasma and lipoprotein α-tocopherols in +E and −E dogs. Plasma concentrations of unlabeled (d_0) and labeled α-tocopherols following oral administration of d_3*RRR*- and d_6*all rac*-α-tocopheryl acetates are shown in Figure 1. At 0 h, plasma d_0-α-tocopherol concentrations in −E dogs were <0.2 nmol/mL; these increased and decreased similarly to the labeled α-tocopherols, but were always less than the deuterated α-tocopherol concentrations. Likely this represents the 200 nmol d_0-α-tocopherol present in the vegetable oil given with the dose of labeled α-tocopherols. By contrast, in +E dogs, d_0-α-tocopherol concentrations were relatively constant throughout the experiment and did not mimic that of the labeled α-tocopherols (Fig. 1).

Plasma-deuterated α-tocopherol concentrations were lower in −E dogs compared with +E dogs. Although the

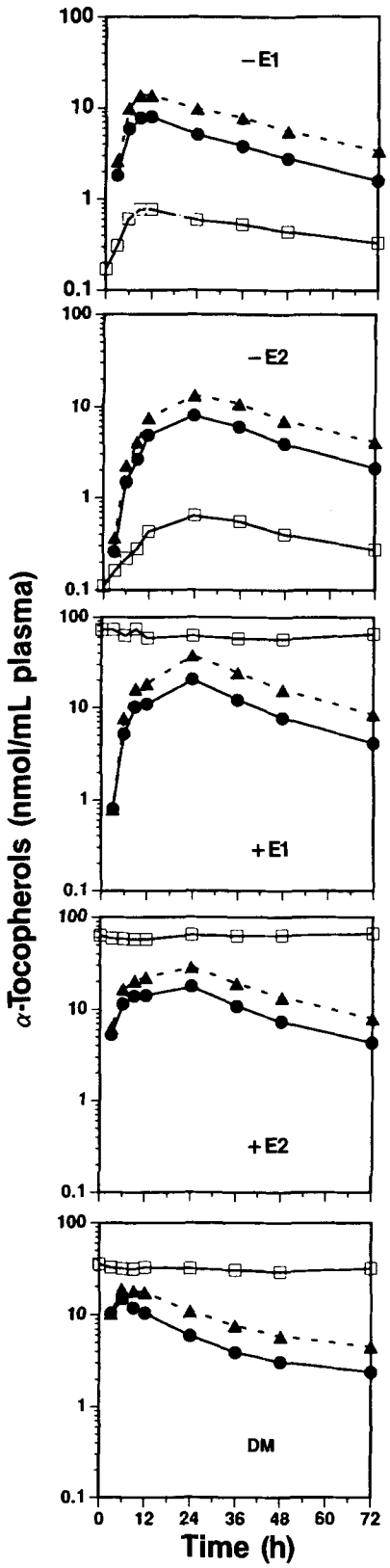

FIG. 1. Plasma concentrations (nmol/mL) of unlabeled (d_0, □), d_3*RRR*-(▲) and d_6*all rac*-α-tocopherols (●) in dogs fed vitamin E-deficient (−E) or vitamin E-supplemented (+E) diets, or in a German Shepherd with degenerative myelopathy (DM) following oral administration of d_3*RRR*- and d_6*all rac*-α-tocopheryl acetates.

shapes of the plasma-deuterated α-tocopherol disappearance curves in the two groups were similar, the areas under the curve of the −E dogs were 42% of those in +E dogs (Table 1). Slopes of the disappearance of portion of plasma *d₃RRR*-α-tocopherol were similar in all dogs (−E1, −0.0234; −E2, −0.0249; +E1, −0.0289; +E2, −0.0256), but maximum observed concentrations (c_{max}) of *d₃RRR* in −E dogs were less than half of those in +E dogs (−E1, 13.8 nmol/mL; −E2, 13.3; +E1, 38.1; +E2, 28.8). The times of maximum observed concentration (t_{moc}) in the plasma were 12 h in −E1 and 24 h in −E2, +E1 and +E2.

Comparison of plasma concentrations of *d₃RRR*- with *d₆all rac*-α-tocopherol in −E and +E dogs shows that vitamin E deficiency did not further enhance the preferential secretion of *RRR*-α-tocopherol into plasma. By 24 h, the plasma in all dogs was preferentially enriched with *d₃RRR*-α-tocopherol; the ratios of *d₃RRR*-/*d₆all rac*-α-tocopherol concentrations at 24 h were 1.88 mol/mol in −E1; 1.63 in −E2; 1.82 in +E1; and 1.61 in +E2. Furthermore, the ratios of the areas under the plasma curves of *d₃RRR*-/*d₆all rac*-α-tocopherol concentrations were also similar in both groups (1.90 in −E1; 1.73 in −E2; 1.90 in +E1; and 1.67 in +E2).

Labeled and unlabeled α-tocopherol contents of the lipoproteins were determined, and representative data are illustrated in Figures 2 and 3 (+E2 and −E2, respectively). (The complete data set is available from the corresponding author upon request.) Unlike human chylomicrons, which contain proportions of tocopherols similar to the administered dose (4,6,7), dog chylomicrons were somewhat enriched in *RRR*- relative to *all rac*-α-tocopherol. The ratios of chylomicron areas under the curves were greater than 1, and in each of the dogs, the c_{max} of chylomicron *d₃RRR*-α-tocopherol was greater than that of *d₆all rac*-α-tocopherol (Figs. 2 and 3). This preferential enrichment of chylomicrons with *RRR*-α-tocopherol could have arisen if dogs have an intestinal tocopherol binding protein, which would function in an analogous manner to the hepatic protein. More likely, dogs may have a faster turnover of chylomicrons with a more rapid VLDL secretion than humans and, therefore, the chylomicrons were contaminated with VLDL. The t_{moc} and c_{max} of the chylomicrons and VLDL support this possibility; in +E1, VLDL t_{moc} (9 h) occurred prior to chylomicron t_{moc} (12 h), and in all dogs, VLDL c_{max} was smaller than chylomicron c_{max} (data not shown).

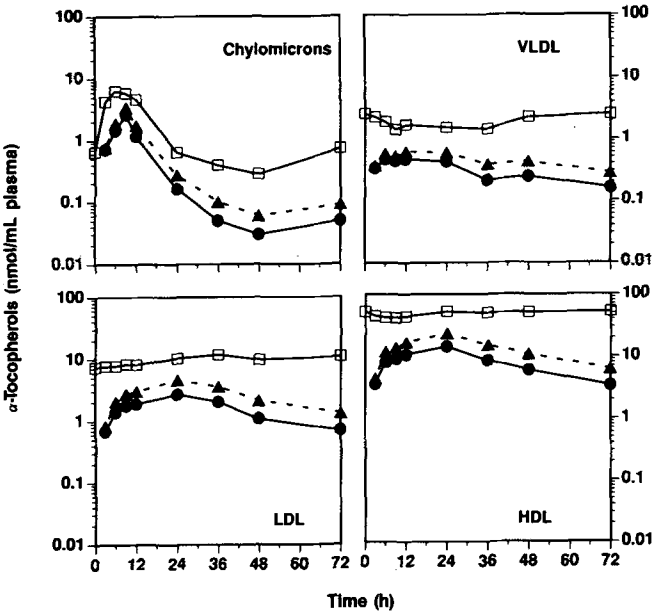

FIG. 2. Lipoprotein concentrations (nmol/mL plasma) of d_0 (□), *d₃RRR*-(▲) and *d₆all rac*-α-tocopherols (●) in dog +E2. Chylomicrons, very low density lipoprotein (VLDL) (d < 1.006 g/mL), low density lipoprotein (LDL) (1.006 < d < 1.063 g/mL) and high density lipoprotein (HDL) (d > 1.063 g/mL) were isolated as described previously (Ref. 4).

It is likely that the preferential enrichment of nascent VLDL with *RRR*-α-tocopherol causes the preferential enrichment of plasma with *RRR*-α-tocopherol because in all dogs (i) the earliest time point (3 h) showed the least enrichment of lipoproteins with *d₃RRR*- compared with *d₆all rac*-α-tocopherol (Figs. 2 and 3); (ii) compared with chylomicrons, other lipoproteins were more dramatically

TABLE 1

Areas Under the Curves (nmol/mL • h) of Deuterated α-Tocopherol Concentrations in Dogs Fed Vitamin E-Deficient (−E) or Vitamin E-Supplemented (+E) Diets, or in a German Shepherd with Degenerative Myelopathy (DM)

Dog	α-Tocopherol label	Plasma	Chylomicrons	VLDL[a]	LDL[a]	HDL[a]
−E1	*d₃RRR*	534	15.6	15.3	128	360
−E2	*d₃RRR*	544	5.7	15.1	107	399
+E1	*d₃RRR*	1351	28.0	24.7	213	1084
+E2	*d₃RRR*	1209	36.8	30.9	190	941
DM	*d₃RRR*	658	47.0	28.9	105	461
−E1	*d₆all rac*	282	9.0	8.7	66	190
−E2	*d₆all rac*	314	3.9	9.3	61	230
+E1	*d₆all rac*	712	17.9	14.3	110	569
+E2	*d₆all rac*	723	25.5	20.4	109	559
DM	*d₆all rac*	392	35.2	19.7	60	262

[a]VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

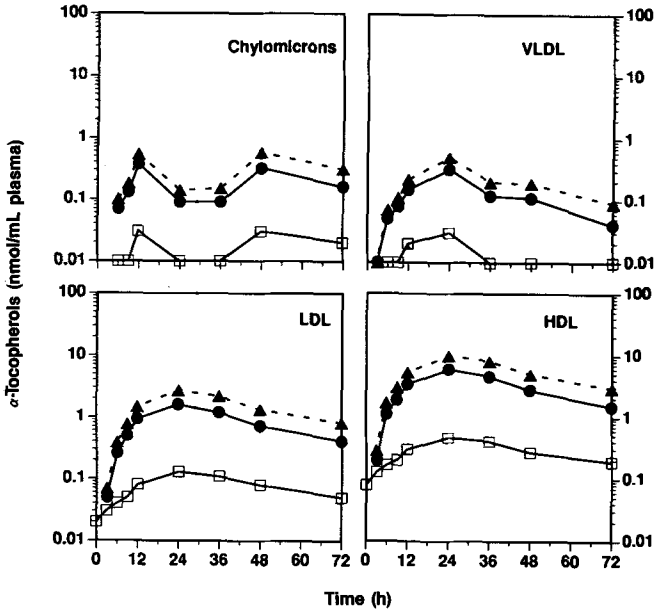

FIG. 3. Lipoprotein concentrations (nmol/mL plasma) of d_0 (□), d_3RRR-(▲) and d_6all *rac*-α-tocopherols (●) in dog −E2. Chylomicrons, VLDL (d < 1.006 g/mL), LDL (1.006 < d < 1.063 g/mL) and HDL (d > 1.063 g/mL) were isolated as described previously (Ref. 4). Abbreviations as in Figure 2.

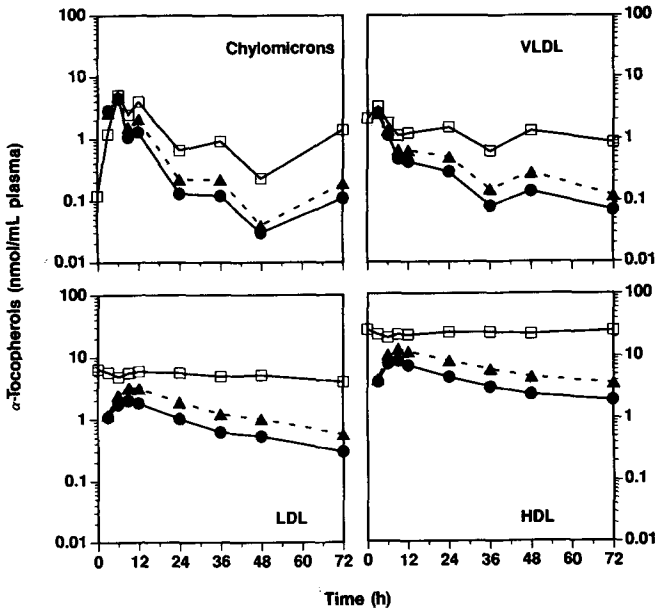

FIG. 4. Lipoprotein concentrations (nmol/mL plasma) of d_0 (□), d_3RRR-(▲) and d_6all *rac*-α-tocopherols (●) in the German Shepherd with degenerative myelopathy. Chylomicrons, VLDL (d < 1.006 g/mL), LDL (1.006 < d < 1.063 g/mL) and HDL (d > 1.063 g/mL) were isolated as described previously (Ref. 4). Abbreviations as in Figure 2.

enriched with *RRR*-α-tocopherol (Figs. 2 and 3 and Table 1); and (iii) VLDL t_{moc} (14 ± 7 h) occurred nearly simultaneously with that of chylomicrons (11 ± 2), but prior to, or simultaneously with, t_{moc} in plasma (21 ± 6 h), LDL (20 ± 8 h) and HDL (21 ± 6).

As might be expected from the plasma data, none of the −E dogs' lipoproteins contained more deuterated tocopherols than did those of the +E dogs (Table 1). The areas under the curves of −E chylomicron d_3RRR-α-tocopherol were about 33% of +E. These areas do not include the second peak in the chylomicron fraction observed in both −E dogs because this peak likely represents enterohepatic circulation and reabsorption of the dose. The areas under the curves of d_3RRR-α-tocopherol in −E dogs' VLDL, LDL and HDL were about 55, 58 and 37% of those of +E dogs, respectively.

Plasma and lipoprotein α-tocopherols in a German Shepherd with DM. Like +E dogs, DM plasma d_0α-tocopherol concentrations following the dose of labeled α-tocopherols were relatively constant throughout the experiment (from 29 to 35 nmol/mL) (Fig. 1). Plasma-deuterated tocopherols in the DM dog peaked earlier than in +E dogs (6 h compared with 21 ± 6, respectively), while the area under the curve of d_3RRR-α-tocopherol was half of that in +E dogs (Table 1). This could be the result of the smaller dose of deuterated tocopherols that was inadvertently administered; however, the kinetic data suggest otherwise. The slope of the disappearance portion of the DM plasma d_3RRR-α-tocopherol curve (−0.0300) was slightly faster than in the other four dogs (−0.0259 ± 0.0024). Furthermore, DM plasma d_3RRR-α-tocopherol peaked at 6 h, earlier than in −E1 (12 h) or −E2, +E1 and +E2 (24 h). Importantly, DM plasma peaked coincident with the DM chylomicron t_{moc} (6 h).

Defective transport of α-tocopherol by DM is also suggested by comparison of the areas under the lipoprotein α-tocopherol curves. The area under the d_3RRR-α-tocopherol curve in DM chylomicrons was 145% of that of +E dogs, and in DM VLDL was about 100%, yet those of DM LDL and HDL were markedly smaller, 52 and 45%, respectively. Despite the reduced incorporation of α-tocopherol into lipoproteins, the ratio of d_3RRR/d_6all *rac* in the plasma at 24 h was 1.91 mol/mol, and the ratio of the plasma areas under the curves was 1.68. The ability to differentiate between α-tocopherols does not necessarily indicate normal function of the tocopherol binding protein because some patients with familial isolated vitamin E deficiency also differentiate between α-tocopherols (7).

DISCUSSION

Vitamin E deficiency in adult beagle dogs in response to a single oral dose of deuterated α-tocopherols did not increase plasma-labeled α-tocopherol concentrations higher than in controls (Fig. 1), did not alter rates of disappearance of plasma labeled α-tocopherols, and did not enhance discrimination between *RRR*- and *all rac*-α-tocopherols (Table 1). Although studies in humans indicate that the tocopherol binding protein is apparently responsible for the discrimination between tocopherols and for the regulation of plasma α-tocopherol (4,6–8), the activity of this protein does not appear to be up-regulated in response to vitamin E deficiency in dogs. This finding may not be as surprising as it first appears. The putative role of the protein is to discriminate between the different forms of dietary vitamin E; therefore, it may already be optimized for recognizing low levels of α-tocopherol. Furthermore, because it is virtually impossible for natural

diets to be vitamin E-deficient, there would have been no evolutionary pressure for the tocopherol binding protein to be responsive to a vitamin E-deficient diet.

Contrary to our expectations, −E dogs had lower plasma-deuterated α-tocopherol concentrations than did +E dogs. If α-tocopherol is readily transferred to vitamin E-depleted tissues, then plasma concentrations may be low. Although measurements of tissue-deuterated α-tocopherols were not carried out, adipose tissue α-tocopherol levels were measured at intervals throughout the two-year vitamin E depletion (20). At 1 and 3 mon prior to sacrifice, adipose tissue α-tocopherol levels were 2.3 ng/mg and 1.6, respectively, in −E1; and 0.6 and 1.0, respectively, in −E2. At autopsy, 1 wk following the deuterated tocopherol study, where a total of 142 mg of α-tocopheryl acetates were consumed, adipose tissue α-tocopherol concentrations had increased to 7.9 ng/mg in −E1 and 2.6 in −E2 (21), suggesting that deuterated tocopherols were transferred from plasma to −E tissues.

From our study, it appears that HDL delivers α-tocopherol to dog tissues. The HDL area under the curve in −E dogs were only 37% of those of +E dogs, while the areas under the curve of −E dog VLDL and LDL were >50% of +E values (Table 1), suggesting that −E HDL readily donated α-tocopherol. This is likely because HDL α-tocopherol readily exchanges to other lipoproteins and to tissues (26–29).

All dogs preferred *RRR*-α-tocopherol compared with *all rac*-α-tocopherol, apparently the result of the preferential secretion of *RRR*-α-tocopherol in VLDL. Unlike humans, dogs have relatively low amounts of apolipoprotein B-containing lipoproteins (25,30–33); therefore, most of the plasma α-tocopherol is in HDL. Nonetheless, enrichment of HDL with *RRR*-α-tocopherol took place after the peak in VLDL d_3*RRR*-α-tocopherol, demonstrating that VLDL enriched with d_3*RRR*-α-tocopherol is the source of HDL d_3*RRR*-α-tocopherol.

We also investigated a German Shepherd dog diagnosed to have DM, in an effort to determine if it had a defective tocopherol binding protein. Dogs afflicted with DM have some, but not all, of the characteristics of familial isolated vitamin E deficiency, a human genetic disorder in vitamin E transport thought to result from a defect in the tocopherol binding protein (7,8,18). Like these patients, this dog did have low adipose tissue α-tocopherol (14 ng/mg wet wt) and peripheral nerve α-tocopherol (13 ng/mg wet wt), but, unlike the patients, the DM plasma vitamin E levels were not markedly reduced, nor did they rapidly decrease (Fig. 4). The labeled vitamin E appeared in the DM plasma apparently as a result of chylomicron, not VLDL catabolism (Fig. 4). However, because both chylomicrons and VLDL turn over rapidly in dogs (30), and because we did not study a control German Shepherd, it is not clear whether or not this dog had an abnormality in vitamin E transport. We had anticipated a much more rapid decline in plasma-deuterated tocopherol concentrations based on studies in humans with familial isolated vitamin E deficiency (7,8,18); however, the relatively low incorporation of α-tocopherol into LDL and HDL is suggestive of a defect in α-tocopherol transport in VLDL. This question must remain unanswered until more dogs with this disorder are studied.

In conclusion, our studies of the transport of vitamin E in dogs have demonstrated that dogs discriminate between *RRR*- and *all rac*-α-tocopherol with a preference for the natural, *RRR*-α-tocopherol form. This differentiation results from the preferential secretion of *RRR*-α-tocopherol in VLDL. Catabolism of VLDL labels the plasma with *RRR*-α-tocopherol, despite the limited amount of apolipoprotein B-containing lipoproteins in dog plasma (25, 30–33). Vitamin E deficiency in dogs does not appear to markedly alter the function of the tocopherol binding protein.

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γ-Tocotrienol as a Hypocholesterolemic and Antioxidant Agent in Rats Fed Atherogenic Diets

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This study was designed to determine whether incorporation of γ-tocotrienol or α-tocopherol in an atherogenic diet would reduce the concentration of plasma cholesterol, triglycerides and fatty acid peroxides, and attenuate platelet aggregability in rats. For six weeks, male Wistar rats (n = 90) were fed AIN76A semisynthetic test diets containing cholesterol (2% by weight), providing fat as partially hydrogenated soybean oil (20% by weight), menhaden oil (20%) or corn oil (2%). Feeding the ration with menhaden oil resulted in the highest concentrations of plasma cholesterol, low and very low density lipoprotein cholesterol, triglycerides, thiobarbituric acid reactive substances and fatty acid hydroperoxides. Consumption of the ration containing γ-tocotrienol (50 mg/kg) and α-tocopherol (500 mg/kg) for six weeks led to decreased plasma lipid concentrations. Plasma cholesterol, low and very low density lipoprotein cholesterol, and triglycerides each decreased significantly ($P < 0.001$). Plasma thiobarbituric acid reactive substances decreased significantly ($P < 0.01$), as did the fatty acid hydroperoxides ($P < 0.05$), when the diet contained both chromanols. Supplementation with γ-tocotrienol resulted in similar, though quantitatively smaller, decrements in these plasma values. Plasma α-tocopherol concentrations were lowest in rats fed menhaden oil without either chromanol. Though plasma α-tocopherol did not rise with γ-tocotrienol supplementation at 50 mg/kg, γ-tocotrienol at 100 mg/kg of ration spared plasma α-tocopherol, which rose from 0.60 ± 0.2 to 1.34 ± 0.4 mg/dL ($P < 0.05$). The highest concentration of α-tocopherol was measured in plasma of animals fed a ration supplemented with α-tocopherol at 500 mg/kg. In response to added collagen, the partially hydrogenated soybean oil diet without supplementary cholesterol led to reduced platelet aggregation as compared with the cholesterol-supplemented diet. However, γ-tocotrienol at a level of 50 mg/kg in the cholesterol-supplemented diet did not significantly reduce platelet aggregation. Platelets from animals fed the menhaden oil diet released less adenosine triphosphate than the ones from any other diet group. The data suggest that the combination of γ-tocotrienol and α-tocopherol, as present in palm oil distillates, deserves further evaluation as a potential hypolipemic agent in hyperlipemic humans at atherogenic risk. *Lipids 28,* 1113–1118 (1993).

That diets rich in polyunsaturated fatty acids lead to reduced plasma cholesterol concentrations and lower atherogenic risk has been well documented (1–7). Studies reported by this laboratory have shown that though menhaden oil (MO) is efficacious in lowering plasma triglycerides (TG), it is also associated with significant increases in plasma fatty acid hydroperoxides (LOPS) and malondialdehyde equivalent substances. Furthermore, such highly polyunsaturated oils lead to depletion of plasma α-tocopherol and other antioxidants. Supplementation of the diet with α-tocopherol significantly increases plasma α-tocopherol concentrations and results in significantly less accumulation of peroxides in the plasma (8). Both epidemiological and clinical data have implicated peroxidized plasma lipid in atherogenesis (9,10). γ-Tocotrienol by itself has been shown in short-term feeding studies to lower total low density lipoprotein (LDL) cholesterol and TGs in both pigs (11) and in humans (12); however, when tested in combination with α-tocopherol, it showed no such effect (13). Neither thiobarbituric acid reactive substances (TBARS) nor LOPS were tested in these trials. Should γ-tocotrienol effectively lower plasma cholesterol or reduce plasma lipid peroxidation, or both, it would be a prime candidate for further testing in humans.

In the present study, γ-tocotrienol alone, or together with α-tocopherol, was tested as a potential hypocholesterolemic agent and also as an antioxidant. For this purpose, γ-tocotrienol, α-tocopherol, or both, were incorporated along with cholesterol into rations fed to rats that provided MO, partially hydrogenated soybean (SO) or corn oil (CO) to test the purported hypocholesterolemic and antithrombotic potential of γ-tocotrienol.

MATERIALS AND METHODS

Ninety male Wistar rats (Hilltop Laboratory Animals, Scottdale, PA) were weighed and divided into nine groups. All rats were fed standard, defined (AIN76A; American Institute of Nutrition) semisynthetic ration (Dyets, Bethlehem, PA) with indicated oils. Commercially available CO and SO were used; MO was obtained from a processor (specially Processed Menhaden Oil; Zapata Haynie Corp., Reedville, VA). After a two-week adaptation period on a chow diet (Ralston-Purina, St. Louis, MO), the animals were fed the semisynthetic rations with the diet treatments for six weeks as described in Table 1. Rats were housed in groups of two or three in plastic cages with access to ration and water *ad libitum*. Room temperature was maintained at 25 ± 2°C. A biphasic lighting cycle, with alternating 12-h light and dark phases, started with the light phase at 7:00 a.m.

The fatty acid composition of the commercial oils used is shown in Table 2. Diets 1–7 provided 18% protein, 42% carbohydrate and 40% fat. The CO ration without added cholesterol (CO − chol; Diet 8) delivered 22% protein, 73% carbohydrate and 5% fat. The SO ration without added cholesterol (SO − chol; Diet 9) contained 17% protein, 43% carbohydrate and 40% fat. Supplemental vitamin E

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Abbreviations: AIN, American Institute of Nutrition; ATP, adenosine triphosphate; chol, cholesterol; CO, corn oil; vitamin E, α-tocopherol; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; LOPS, fatty acid hydroperoxides; MO, menhaden oil; SO, partially hydrogenated soybean oil; TBARS, thiobarbituric acid reactive substances; TG, triglycerides; VLDL, very low density lipoprotein.

TABLE 1

Composition of Experimental Diets[a]

Diet number	Oil[b]	Cholesterol	TT[c]	α-T[d]
1	SO	+	−	−
2	SO	+	+	−
3	MO	+	−	−
4	MO	+	+	−
5	MO	+	−	+
6	MO	+	+	+
7	MO	+	++	−
8	CO	−	−	−
9	SO	−	−	−

[a]All diets contained salts and vitamins. AIN76A (g/kg): casein (200); DL-methionine (3); cornstarch (110); sucrose (370); CO − chol: cornstarch (157), sucrose (523); SO − chol: cornstarch (115), sucrose (385); cellulose (10); salts (30); vitamins (10); choline bitartrate (2); oil (20 or 200). Salts: calcium phosphate, dibasic (500.0); sodium chloride (74.0); potassium citrate, monohydrate (220); potassium sulfate (52.0); magnesium oxide (24.0); manganous carbonate (3.5); ferric citrate (6.0); zinc carbonate (1.6); cupric carbonate (0.3); potassium iodate (0.01); sodium selenite (0.5); chromium potassium sulfate (0.55); sucrose (118.03). Vitamins: thiamin-HCl (0.6); riboflavin (0.6); pyridoxine HCl (0.7); niacin (3.0); calcium pantothenate (1.6); folic acid (0.2); biotin (0.02); vitamin B-12, 0.1% (1.0); retinyl palmitate, 500,000 IU/g (0.8); vitamin D_3, 400,000 IU/g (0.25); tocopheryl acetate, 500 IU/g (10.0); menadione sodium bisulfite (0.08); sucrose (981.15).

[b]SO, partially hydrogenated soybean oil, 20% (by weight); MO, menhaden oil, 20%; CO, corn oil, 2%; chol, cholesterol, 2%.

[c]γ-Tocotrienol: +, 50 mg/kg; ++, 100 mg/kg.

[d]dl-α-Tocopherol: 500 mg/kg.

TABLE 2

Fatty Acid Composition of Dietary Fats Fed to Rats During Six-Week Feeding Studies[a]

Fatty acid	(wt%)		
	Soy oil	Corn oil	Menhaden oil
14:0	0.1		9.0
15:0			0.7
16:0	8.9	13.0	17.1
16:2			1.7
16:3			1.7
16:4			1.8
17:0			0.9
18:0	3.9	2.5	2.8
18:1	47.2	25.0	11.4
18:2	36.7	58.0	1.5
18:3	2.7	1.0	1.6
18:4			3.4
20:0	0.3		0.2
20:1	0.1	0.5	1.6
20:4			2.0
20:5			15.5
21:5			0.8
22:0	0.2		
22:1			0.4
22:5			2.4
22:6			9.1

[a]Soy oil was partially hydrogenated soybean oil containing vitamin E, 200 mg/kg (Procter & Gamble, Cincinnati, OH). Corn oil contained vitamin E, 112 mg/kg (CPC International, Elizabeth, NJ). Menhaden oil contained vitamin E, 50 mg/kg (Zapata Haynie Corp., Reedville, VA).

(>96% dl-α-tocopherol; Roche, Nutley, NJ) and γ-tocotrienol (>88% pure by reverse-phase chromatography; PORIM, Kuala Lumpur, Malaysia) were mixed with the dietary oil before being added to the diet. Diets were made fresh weekly and were flushed with nitrogen and stored in daily allotments in "zip-loc" polethylene bags at −20 ± 2°C.

Diets provided adequate levels of vitamins. Calculations indicated that the diets provided ample thiamin and riboflavin (1.3 mg/1000 kcal each) for the dietary energy load, pyridoxine for the protein load (7.0 mg/4 g protein/d) and vitamin E for the fat load, there being more than 0.4 IU of α-tocopherol per g linoleic acid equivalent in all diets.

At the end of the study, the rats were fasted overnight (18 h). The animals were weighed, then euthanized with carbon dioxide. All animals were bled by syringe from the dorsal aorta. Two and one-half mL of blood was transferred to a sterile, citrate-containing Vacutainer tube (0.105 M buffered sodium citrate; Becton-Dickinson, Rutherford, NJ). The remaining 4.5 mL of blood was transferred to an ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer tube (0.05 mL of 0.34 M tripotassium EDTA; Becton-Dickinson) for lipid analysis. After centrifugation to pellet the red cells, the plasma was transferred to a screw-cap polyethylene tube, flushed with nitrogen and frozen until analysis.

Plasma cholesterol, high-density lipoprotein (HDL) cholesterol and TGs were determined using a dry film kit (Eastman Kodak, Rochester, NY; C303-C341). The kits were used according to the manufacturer's instructions. Plasma oxidation products were measured spectrophotometrically as TBARS by the method of Mihara *et al.* (14) against 1,1,3,3-tetramethoxypropane as reference. Plasma LOPS were measured spectrophotometrically with a kit (LPO; Kamiya Biomedical, Thousand Oaks, CA), according to the manufacturer's instructions. The sample LOPS convert 10-*N*-methylcarbamoyl-3,7-dimethylamino-10H-phenothiazine to methylene blue in equimolar quantity. They were assayed with cumene hydroperoxides as reference as described by Ohishi *et al.* (15). Absorbance data were obtained with a UV-vis spectrophotometer (Milton Roy Company, Rochester, NY). Plasma retinol and α-tocopherol were separated on a C18 column by the high-performance liquid chromatography (HPLC) method of Bieri *et al.* (16), which was modified to detect analytes near their absorbance maxima—retinol at 325 nm, α-tocopherol at 300 nm and tocopheryl acetate (the internal standard) at 286 nm. All manipulations were done under amber light to reduce photo-induced oxidation. Solvents (HPLC grade) were obtained from EM Separations (Gibbstown, NJ).

Platelet response was measured with an impedance aggregometer (Chronolog, Havertown, PA) by the method of Galvez *et al.* (17) and Mackie *et al.* (18). Whole blood was incubated with the agonist for 6 min, at which time the response was recorded. Reagents were obtained from Sigma Chemical Company (St. Louis, MO), except for luciferin-luciferase and collagen for platelet studies (Chronolog, Havertown, PA) and dl-α-tocopheryl acetate (Roche), unless indicated otherwise.

Group means and standard errors were analyzed by Student's *t*-test. Past studies had shown that at n = 10, statistical significance at $P < 0.05$ can be achieved with 95% confidence.

RESULTS

At the start of the feeding trials, the mean body weights of the animals in all nine groups was 173 ± 2.7 g, and after six weeks of feeding, the average body weights for each group ranged from 416–473 g. All diets were well accepted; no significant difference was observed in mean body weights between groups, except for a borderline increase in the SO − chol group (Diet 9). No signs of vitamin deficiency on snout, paws or tail were observed; no hemolysis or xerophthalmia was seen. Growth rates and coat sheen and quality suggested that the diets were adequate for maintaining normal growth and vigor.

Plasma lipid data that was measured after the animals were kept on the test diets for six weeks are shown in Table 3. Animals given 20% MO with 2% cholesterol (MO/chol; Diet 3) had the highest total cholesterol levels when compared to all other groups, including controls and Diets 8 and 9. In animals fed the MO/chol diet with α-tocopherol and γ-tocotrienol (Diet 6), the lowest cholesterol levels were observed, which were significantly lower than those of animals fed the MO/chol diet with α-tocopherol (Diet 5). They were also somewhat lower than those of the group fed MO/chol with γ-tocotrienol (Diet 4). Diet 4 also resulted in a plasma cholesterol concentration that was significantly lower than that of the CO control without added cholesterol (Diet 8), 53.5 ± 4.4 *vs.* 72.8 ± 5.1, $P < 0.01$. In the 20% SO groups with 2% cholesterol (SO/chol; Diets 1 and 2), plasma cholesterol concentrations did not decrease significantly with γ-tocotrienol supplement, 75.7 *vs.* 66.9, although plasma cholesterol decreased without the 2% cholesterol supplement in the diet, SO/chol *vs.* SO − chol, 75.7 *vs.* 57.2 (Diet 1 *vs.* 9).

Plasma combined LDL and very low density lipoprotein (VLDL) cholesterol concentrations approximated the values found for total cholesterol (Table 3). The MO/chol group (Diet 3) had the highest total and LDL + VLDL cholesterol levels; the lowest LDL + VLDL concentrations were measured in the groups fed γ-tocotrienol supplement (Diets 4 and 7). No dosage effect was observed (Diets 4 and 7). The addition of α-tocopherol did not further enhance the hypocholesterolemic effect of γ-tocotrienol. No dietary supplement resulted in total or LDL + VLDL cholesterol levels as low as those in the group not given cholesterol, which was also reflected in the higher HDL cholesterol found in this group (SO − chol, Diet 9). Incorporation of γ-tocotrienol in the SO/chol ration resulted in a lower LDL + VLDL cholesterol concentration (Diet 1 *vs.* 2); however, omission of the dietary cholesterol was of a much larger effect (Diet 1 *vs.* 9).

Supplementary γ-tocotrienol did not result in increased HDL cholesterol concentrations (Table 3). Plasma HDL cholesterol concentrations were lower in animals fed the 2% cholesterol supplement, compared with both the CO − chol group (Diet 8) and the SO − chol (Diet 9) group. The group fed MO/chol had a mean HDL cholesterol concentration statistically higher than all of the other groups, except those fed added α-tocopherol (Diet 5). γ-Tocotrienol had a negligible effect when added to the MO/chol groups; the combined α-tocopherol (500 mg/kg) with γ-tocotrienol group (Diet 6), and 100 mg/kg γ-tocotrienol group (Diet 7) had statistically lower values than Diets 3 or 8. In general, higher plasma HDL cholesterol was associated with lower LDL + VLDL cholesterol concentrations.

In the case of plasma TGs, the SO/chol groups (Diets 1 and 2) and MO/chol (Diet 3) group produced concentrations similar to the CO − chol (Diet 8) and the SO − chol groups (Diet 9). In the SO/chol groups, γ-tocotrienol supplementation did not attenuate the plasma TG concentration (Diet 1 *vs.* 2); the cholesterol supplementation masked a γ-tocotrienol effect, if any. In contrast, the MO/chol groups inclusion of γ-tocotrienol or α-tocopherol resulted in significantly improved plasma TG clearance, or decreased synthesis, or both (Diets 3, 4, 5, 6 and 7). A combination of α-tocopherol and γ-tocotrienol was not better than either alone (Diet 3 *vs.* 4, 5 or 6). γ-Tocotrienol at 100 mg/kg was no more effective than at 50 mg/kg in influencing plasma TG concentrations (Diet 4 *vs.* 7). The groups fed MO/chol with or without α-tocopherol or γ-tocotrienol (Diets 4, 5, 6 and 7) had lower TG concentrations than the other groups, pointing to an effect of the acyl composition in addition to the γ-tocotrienol effect.

Oxidation product levels measured as TBARS or LOPS varied both with the dietary oil fed and the antioxidant supplement (Table 4). As expected, the TBARS were

TABLE 3

Plasma Cholesterol and Triglycerides (TG) Concentrations of Rats Fed Atherogenic Diets for Six Weeks with or without Tocotrienol or Tocopherol[a]

Diet number	Diet[b]	Cholesterol	LDL + VLDL Chol	HDL Chol	TG
1	SO	57.7 ± 12.1[d]	48.5 ± 8.5[d]	16.7 ± 1.3[d]	65.5 ± 13.6
2	SO + TT	66.9 ± 5.0[d]	35.8 ± 3.6[c,d]	19.0 ± 1.5[d,e]	76.1 + 10.0[d]
3	MO	112 ± 6.4[c,e]	79.6 ± 4.7[c,e]	23.1 ± 1.6[c]	51.1 ± 5.4
4	MO + TT	61.3 ± 6.3[d]	36.0 ± 4.2[d]	20.3 ± 1.3[c,e]	28.7 ± 2.8[c,d,e]
5	MO + E	67.9 ± 3.8[d]	42.3 ± 2.9[d]	21.6 ± 1.4	24.9 ± 3.3[c,d,e]
6	MO + E + TT	53.5 ± 4.4[c,d,e]	30.8 ± 3.4[c,d]	18.5 ± 1.5[d,e]	26.3 ± 4.2[c,d,e]
7	MO + 2TT	57.2 ± 7.0[d]	32.6 ± 5.1[d]	20.0 ± 1.3[d,e]	28.8 ± 5.4[c,d,e]
8	CO − chol	72.8 ± 5.1[d]	34.4 ± 4.3[d]	29.2 ± 1.7[c,d]	57.7 ± 4.6
9	SO − chol	57.2 ± 5.1[d,e]	20.7 ± 4.2[c,d,e]	28.9 ± 0.8[c,d]	47.2 ± 3.0

[a]Mean ± SEM, n = 10 animals/group. See Table 1 for abbreviations. LDL, low density lipoprotein; VLDL, very low density lipoproteins; HDL, high density lipoprotein.
[b]See Table 1 for details.
[c]$P < 0.05$ *vs.* Diet 1.
[d]$P < 0.05$ *vs.* Diet. 3.
[e]$P < 0.05$ *vs.* Diet. 8.

TABLE 4

Plasma TBARS and LOPS Data of Rats Fed Atherogenic Diets for Six Weeks with or without Tocotrienol or Tocopherol[a]

Diet no.	Diet[b]	μM TBARS[c]	μM LOPS[d]
1	SO	2.09 ± 0.2[g]	6.01 ± 1.6
2	SO + TT	3.59 ± 1.4[f]	7.22 ± 2.3
3	MO	11.0 ± 1.8[e,g]	6.86 ± 1.0
4	MO + TT	3.02 ± 0.9[f]	3.75 ± 1.0[f]
5	MO + E	6.21 ± 0.5[f,g]	5.33 ± 0.9
6	MO + E + TT	3.93 ± 1.0[f,g]	4.17 ± 0.7[f]
7	MO + 2TT	4.38 ± 0.7[e,f,g]	4.26 ± 0.6[f]
8	CO − chol	0.76 ± 0.3[e,f]	8.91 ± 2.1[f]
9	SO − chol	1.56 ± 0.5[f]	5.73 ± 1.1

[a]Mean ± SEM, n = 10 animals/group. Abbreviations as in Table 1.
[b]See Table 1 for details.
[c]Thiobarbituric acid reactive substances.
[d]Fatty acid hydroperoxides.
[e]$P < 0.05$ *vs.* Diet 1.
[f]$P < 0.05$ *vs.* Diet 3.
[g]$P < 0.05$ *vs.* Diet 8.

greatest in the MO/chol group without α-tocopherol or γ-tocotrienol supplement (Diet 3), presumably because of the high level of unsaturation in the MO-rich diet. γ-Tocotrienol inclusion in the SO/chol ration had no measurable effect on either TBARS or LOPS (Diet 1 *vs.* 2). α-Tocopherol supplementation (Diet 5) resulted in moderately decreased TBARS concentrations, but when γ-tocotrienol was included, either alone (Diets 4 or 7) or combined with α-tocopherol (Diet 6), TBARS values decreased significantly as compared with the MO/chol group lacking a supplement of either antioxidant (Diet 3). α-Tocopherol in combination with γ-tocotrienol was no more effective than the latter alone in keeping TBARS concentrations down (Diet 3 *vs.* 4, 5 and 6). Twice the amount of γ-tocotrienol did not reduce TBARS levels any further (Diet 4 *vs.* 7); no dose-response effect was apparent. The lowest TBARS concentrations were measured in the groups without the 2% cholesterol supplement (Diets 8 and 9). Although TBARS levels were lower in the SO and CO than the MO/chol groups (Diets 1, 2, 8 and 9 *vs.* Diets 3, 4, 5, 6 and 7), the SO/chol group with γ-tocotrienol was somewhat higher than SO − chol (Diet 2 *vs.* 9). This occurred in spite of the inclusion of 50 ppm of α-tocopherol in the MO. The CO group (Diet 8) contained the least TBARS; it also contained the least fat, 2% rather than 20%, and 112 ppm of α-tocopherol.

Some oxidation to hydroperoxides occurred for each oil fed, even for the CO − chol group as seen in the LOPS data (Diets 1, 3 and 8; Table 4). The LOPS concentrations were similar in groups with or without the 2% cholesterol supplementation (Diet 1 *vs.* 9). Although γ-tocotrienol was not effective in protecting the SO/chol groups (Diet 1 *vs.* 2), it effectively protected the MO/chol groups (Diets 3, 4, 6 and 7), and α-tocopherol did not afford such protection in these trials (Diet 3 *vs.* 5). No dose-response effect was seen under these conditions for γ-tocotrienol (Diet 4 *vs.* 7). Also, in the case of LOPS, as for TBARS, no apparent synergism of α-tocopherol with γ-tocotrienol was observed (Diets 3 *vs.* 4, 5 or 6), the combination being no better than γ-tocotrienol alone at 50 mg/kg.

Supplementation of the SO/chol group with γ-tocotrienol at 50 mg/kg (Diet 1 *vs.* 2) did not result in increased plasma α-tocopherol concentrations (Table 5). The lowest plasma concentration of α-tocopherol was measured in the MO/chol group (Diet 3) fed very polyunsaturated oil. Inclusion of 50 mg/kg of γ-tocotrienol had no effect on plasma α-tocopherol (Diet 4), but when that amount was doubled (Diet 7), the α-tocopherol concentration increased significantly, $P < 0.05$. Yet this level was still lower than when α-tocopherol was added alone or in combination with γ-tocotrienol at 50 mg/kg (Diets 5 and 6 *vs.* Diet 7). Plasma α-tocopherol was highest in the CO − chol group (Diet 8), being comparable to the SO groups (Diets 1, 2 and 9).

Plasma retinol concentrations were the lowest in the MO/chol group supplemented with α-tocopherol (Diet 5) (Table 5). The highest levels of plasma retinol measured occurred in groups not given cholesterol (Diets 8 and 9). In the SO/chol groups, γ-tocotrienol apparently spared plasma retinol (Diet 1 *vs.* 2). In the MO/chol groups, γ-tocotrienol at 100 mg/kg or α-tocopherol combined with γ-tocotrienol at 50 mg/kg resulted in higher retinol concentrations (Diet 3 *vs.* 6 and 7).

Using collagen as agonist, the only significant difference observed in platelet aggregation responses occurred between the α-tocopherol-supplemented group (Diet 5) and the SO − chol group (Diet 9), as compared with the CO − chol group (Diet 8) (Table 6). Inclusion of cholesterol supplement resulted in hyperaggregable platelets, as seen in the SO/chol and SO − chol groups (Diet 1 *vs.* 9). Neither γ-tocotrienol nor α-tocopherol at the levels fed significantly decreased platelet aggregation in the SO/chol or the MO/chol groups (Diet 1 *vs.* 2, and Diet 3 *vs.* 4, 5, 6 and 7).

Neither γ-tocotrienol nor α-tocopherol supplementation decreased platelet adenosine triphosphate (ATP) release with thrombin as agonist (Diet 1 *vs.* 2 and Diet 3 *vs.* 4, 5, 6 or 7; Table 5). However, the acyl composition, particularly the ω3 content, resulted in reduced platelet ATP charge. The MO/chol group without α-tocopherol or γ-tocotrienol showed significantly less ATP release, presumably indicative of less ATP platelet charge. ATP release of the other groups were virtually identical and greater than Diet 3. Supplementation with either α-tocopherol or γ-tocotrienol resulted in increased ATP release in the MO/chol groups (Diet 3 *vs.* 4, 5, 6 and 7),

TABLE 5

Plasma α-Tocopherol and Retinol Concentrations of Rats Fed Atherogenic Diets for Six Weeks with or without Tocotrienol or Tocopherol[a]

Diet number	Diet[b]	α-Tocopherol (mg/dL)	Retinol (μg/dL)
1	SO	3.46 ± 0.78[d]	76 ± 18[e]
2	SO + TT	3.03 ± 0.71[d]	123 ± 44[c]
3	MO	0.60 ± 0.21[c,e]	71 ± 27[e]
4	MO + TT	0.51 + 0.26[c,e]	80 ± 25[e]
5	MO + E	2.72 ± 0.72[d]	48 ± 8[c,d,e]
6	MO + E + TT	2.90 ± 0.84[d]	110 ± 25[d]
7	MO + 2TT	1.34 ± 0.40[d,e]	102 ± 22[c,d]
8	CO − chol	3.55 ± 0.85[d]	189 ± 29[d]
9	SO − chol	3.52 ± 0.84[d]	170 ± 31[d]

[a]Mean ± SEM; n = 10 animals/group. Abbreviations as in Table 1.
[b]See Table 1 for details. [c]$P < 0.05$ *vs.* Diet 1. [d]$P < 0.05$ *vs.* Diet 3.
[e]$P < 0.05$ *vs.* Diet 8.

TABLE 6

Platelet Aggregation Results of Rats Fed Atherogenic Diets for Six Weeks with or without Tocotrienol or Tocopherol[a]

Diet number	Diet[b]	Aggregation (Ω)[c]	ATP release (μM)[d]
1	SO	10.8 ± 2.3	1.69 ± 0.14[f]
2	SO + TT	7.6 ± 1.3	1.74 ± 0.03[f]
3	MO	10.8 ± 2.3	0.92 ± 0.14[e,g]
4	MO + TT	8.1 ± 0.4	1.54 ± 0.26[f]
5	MO + E	7.4 ± 0.9[g]	1.76 ± 0.24[f]
6	MO + E + TT	8.4 ± 1.3	1.75 ± 0.25[f]
7	MO + 2TT	8.6 ± 1.2	1.46 ± 0.11[f]
8	CO − chol	10.1 ± 0.9	1.70 ± 0.14[f]
9	SO − chol	6.7 ± 0.4[g]	1.38 ± 0.14[f]

[a]Mean ± SEM, n = 10 animals/group. Abbreviations as in Table 1. ATP, adenosine triphosphate.
[b]See Table 1 for details.
[c]Ω, ohms; Aggregation measured after six minutes; equine collagen, 2 mg/mL final concentration.
[d]Thrombin (human plasma) agonist, 1 NIH U/mL final concentration.
[e]$P < 0.05$ *vs.* Diet 1.
[f]$P < 0.05$ *vs.* Diet 3.
[g]$P < 0.05$ *vs.* Diet 8.

possibly related to lower plasma hydroperoxide levels (Table 4).

DISCUSSION

Incorporation of dietary cholesterol in these test rations (Diets 1-7) was expected to result in elevated plasma cholesterol; such was observed in the SO/chol groups (Diet 1 *vs.* 9); Table 3). It is presumed that the presence of *trans*-fatty acid in the SO groups led to elevated plasma cholesterol. Lichtenstein *et al.* (19) have observed this when feeding human subjects partially hydrogenated CO *vs.* CO. Our data confirmed that as dietary fatty acid unsaturation increased, plasma cholesterol decreased (Diet 8 *vs.* 9); however, the MO/chol group without chromanol supplement (Diet 3) had the highest plasma cholesterol. Though dietary MO has been reported by Haglund *et al.* (20) to attenuate plasma TG levels, its hypocholesterolemic effect, if any, was marginal.

Supplementation of these test diets with 50 mg/kg of γ-tocotrienol resulted in reduced total and LDL + VLDL cholesterol (Table 3). Marked decreases were measured in the MO/chol groups when either γ-tocotrienol or α-tocopherol was added (Diets 2, 4 and 5 *vs.* 3). γTbcotrienol at 100 mg/kg of diet spared α-tocopherol, but no further decrease in total or LDL + VLDL cholesterol occurred. That no further decrease in plasma cholesterol occurred at this higher dosage suggested that key 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase sites were approaching saturation at the lower dosage. No significant decrease of plasma cholesterol was observed in the SO/chol groups with supplementation of γ-tocotrienol, 50 mg/kg (Diet 1 *vs.* 2). The *trans*-fatty acid content of the SO/chol and SO − chol diets likely introduced matrix ordering incompatible with access to HMG-CoA reductase by γ-tocotrienol. As in previous rat studies done in this laboratory (8,21), supplementation of rat diets with 20% MO and 2% cholesterol produced the highest total and LDL + VLDL cholesterol concentrations.

The finding that the combination of α-tocopherol and γ-tocotrienol produced further decreases over γ-tocotrienol alone was somewhat unexpected, as in an earlier human study, Qureshi *et al.* (11) had found a subgroup which did not respond to a mixture of tocopherols and tocotrienols (although the majority did). Only when they were supplemented with 200 mg/d of γ-tocotrienol for an additional 14-28 d did their total and LDL cholesterol decrease. Wahlquist *et al.* (13) has also reported a similar failure of human subjects to respond to a mixture of tocopherols and γ-tocotrienol (obtained from the γ-tocotrienol-rich fraction of palm oil). The lower dosage used by Qureshi *et al.* (12) may have explained the different response. In those studies the maximal dosage fed was about 2.5 mg/kg/d as mixed tocotrienols; in this study we fed γ-tocotrienol at a level of about 2.5 mg/kg/d. Hence, γ-tocotrienol may be a more efficacious hypocholesterolemic agent than the mixed tocotrienols. If future studies show the combination of γ-tocotrienol and α-tocopherol to be as effective, or more effective than γ-tocotrienol alone, then supplying a naturally occurring mixture would be easier and more cost-effective.

As to the method of action of γ-tocotrienol, animal and tissue culture studies (22,23) have shown that γ-tocotrienol derived from barley or palm oil lowers serum cholesterol concentrations by inhibiting enzymes of the cholesterol biosynthetic pathway, including HMG-CoA reductase and cholesterol-7-hydroxylase. The currently widely used HMG-CoA reductase inhibitors of the lovastatin family are effective in similarly reducing serum lipids percentagewise (24,25), but they have no activity against free radicals and are quite expensive on a long-term basis.

Neither γ-tocotrienol nor α-tocopherol supplementation altered HDL cholesterol concentrations. HDL concentrations were higher in groups not given exogenous cholesterol (Diets 8 and 9) as compared with each of the other groups (Diets 1-7) fed rations with cholesterol (2%) along with SO, CO, or low erucic acid rapeseed oil (8,21). The apparent lack of a chromanol effect on HDL cholesterol levels suggests that the effect observed was related more directly to LDL and/or VLDL clearance, *i.e.*, membrane dynamics dependent upon acyl composition or stacking order, because in groups with higher LDL + VLDL concentrations, HDL cholesterol levels were lower (Diets 1, 2, 3 and 5 *vs.* 4, 6, 7, 8 and 9).

Supplementation with γ-tocotrienol did not result in decreased plasma TGs in the SO/chol groups (Diet 1 *vs.* 2), presumably a reflection of higher melting points, hence order, in the membranes of these groups, as also seen by the addition of the cholesterol supplement (Diet 1 *vs.* 9). In contrast, in MO/chol groups given γ-tocotrienol or α-tocopherol (Diets 4-7), the plasma TG concentrations decreased significantly; whether this reflected altered synthesis or clearance because of altered membrane dynamics is unclear.

As increased unsaturation of lipid increases susceptibility to oxidation of lipid *ex vivo*, so plasma lipid peroxidation increased with increasing dietary lipid unsaturation (Table 4). Plasma TBARS and LOPS accumulated at higher concentrations in groups fed more highly unsaturated lipid, as expected: CO, SO and MO. Supplementation with α-tocopherol (500 mg/kg) was accompanied by a decrease in malondialdehyde equivalent material. However, γ-tocotrienol supplementation (50 mg/kg) netted the

lowest concentrations of hydroperoxides and substances reactive with TBA. The trend observed in each diet for LOPS was similar, though greater quantitatively, for each group. The hydroperoxides level for CO − chol was distinctly greater than the TBA adducts concentration. These data suggest that γ-tocotrienol may be a more efficient antioxidant than α-tocopherol on a molar basis.

Much work in recent years has suggested an important inverse relationship between the development of arteriosclerosis and plasma antioxidant concentrations. This was well stated by Riemersma *et al.* (26), who showed a clear, inverse relation between the incidence of angina pectoris in men with low serum vitamin E levels. They also demonstrated that this relationship was a better predictor of disease development than serum cholesterol, hypertension or smoking. In this study, the addition of α-tocopherol improved the plasma malondialdehyde equivalent material concentrations, and in combination with γ-tocotrienol, these levels were decreased to levels similar to the SO groups, which were fed lower levels of unsaturated fat. The plasma LOPS concentrations followed a similar trend, though the significant decreases were observed in the groups with γ-tocotrienol supplementation. It appears, therefore, that a dual benefit, lowering of plasma cholesterol and TG as well as peroxides, may be achieved by supplementation with γ-tocotrienol in combination with α-tocopherol.

Further, as in previous studies (8,21), plasma α-tocopherol concentrations decreased when highly unsaturated oils, such as MO, were fed (Table 5). With a dietary fat load of 20% MO, γ-tocotrienol at 50 mg/kg did not improve the plasma α-tocopherol economy. However, if incorporated at a level of 100 mg/kg, γ-tocotrienol spared plasma α-tocopherol. With the combination of supplementary γ-tocotrienol and α-tocopherol, the highest plasma levels of α-tocopherol were measured.

In spite of significant hypocholesterolemic and hypotriglyceridemic effects of γ-tocotrienol, no significant antiaggregation effect of this tocotrienol on platelets was measured at either supplementation level. The attenuation of platelet aggregation to collagen agonist in the group supplemented with α-tocopherol (Diet 5) was similar to that seen in human feeding studies in this laboratory (27). The platelet effect of γ-tocotrienol would be expected to be similar to that of α-tocopherol; however, neither of the two levels of γ-tocotrienol tested was sufficient to reduce the platelet response to collagen in these short-term trials. The geometry of the unsaturated side chain of γ-tocotrienol may restrict its partitioning into platelet membranes compared with α-tocopherol. ATP release after thrombin stimulation of the MO/chol group without chromanol supplement (Diet 3) was of the same order of response as seen in another study from this laboratory (21); it showed no further improvement with either γ-tocotrienol or α-tocopherol supplementation.

In summary, supplementation with γ-tocotrienol alone or in combination with α-tocopherol resulted in decreased concentrations of plasma total and LDL + VLDL cholesterol, TG, TBARS and LOPS, and raised plasma α-tocopherol concentrations in these feeding studies with semisynthetic, hypercholesterolemic diets. Platelet hyperaggregability was decreased by α-tocopherol supplementation. No noxious effect was observed in any of the groups supplemented with γ-tocotrienol. The combination of γ-tocotrienol with α-tocopherol was better, in general, than either supplement alone; hence, the combination of α-tocopherol with γ- (or other) tocotrienols deserves further evaluation as a potential antiatherogenic agent.

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Inhibition by Cardiolipins of Platelet-Activating Factor-Induced Rabbit Platelet Activation

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Evidence is presented that cardiolipin, a naturally occurring phospholipid, inhibits the aggregatory effect of platelet-activating factor (paf) on rabbit platelets *in vitro*. Bovine heart cardiolipin was shown to inhibit the aggregation of washed rabbit platelets induced by 1×10^{-10} M and 2×10^{-10} M paf with IC_{50} values (doses for half-maximal inhibition) of $8.4 \pm 0.8 \times 10^{-7}$ M and $2.6 \pm 0.6 \times 10^{-6}$ M, respectively. Phosphonocardiolipin was also able to inhibit platelet aggregation induced by 1×10^{-10} M paf with an IC_{50} value of $3 \pm 1 \times 10^{-7}$ M. Both compounds, in concentrations up to 1×10^{-5} M, were unable to aggregate washed rabbit platelets and failed to inhibit the aggregation induced by 0.9 and 1.8 μM adenosine diphosphate or 0.2–1.0 μM arachidonic acid. By contrast, the acetylated derivative of cardiolipin exerted an aggregatory effect on aspirin-treated rabbit platelets in the presence of creatine phosphate/creatine phosphokinase. This aggregation was inhibited by the specific paf antagonists BN 52021 and WEB 2086. Also, platelets treated with acetyl-cardiolipin were insensitive to the aggregatory effect of paf. Phosphatidic acid, phosphatidylglycerol, *bis*(dipalmitoylglycero)phosphate and their phosphono analogues were totally inactive. Similar data were obtained when platelet-rich plasma was used instead of washed rabbit platelets. Our results support the hypothesis that the effect of cardiolipin is mediated through specific paf receptors that act on the rabbit platelet membrane. *Lipids 28,* 1119–1124 (1993).

Platelet-activating factor (paf), 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1–3), is a naturally occurring lipid mediator with a wide range of effects *in vivo* and *in vitro*. These effects include vasodilation and hypotension (3–5), increased vascular permeability (4,6), a negative inotropic effect, coronary vasoconstriction, reduced coronary blood flow (7–11), cardiac arrhythmias (11) and bronchoconstriction (12,13). In addition, paf can stimulate various cells, such as platelets, neutrophils, macrophages, endothelial cells and protozoan cells (14–16). It has also been suggested that paf is involved in the pathogenesis of various diseases, such as bronchial asthma (17), endotoxemia (18), acute serum sickness (19,20), ischemic bowel necrosis (21), gastro-intestinal ulceration (22), cold urticaria (23) and psoriasis (24).

Over the last few years, various synthetic and naturally occurring compounds have been identified that act as specific paf antagonists (25). These include compounds that are structural analogs of paf and others with no structural resemblance. Endogenous lipid inhibitors of paf have been found in various mammalian tissues and organs (26–28). Phospholipids with a chromatographic behavior similar to that of cardiolipins and phosphatidylglycerols, which specifically inhibit rabbit platelet aggregation induced by paf *in vitro*, have been isolated from perfusates of guinea pig heart (Tsoukatos, D., Chignard, M., Boullet, C., LeCoedic, J.P., and Benveniste, J., unpublished data).

In the present study, we examined the effect of bovine heart cardiolipin, phosphatidylglycerol and of some structural analogues, on rabbit platelet aggregation induced by paf and other agonists *in vitro*.

MATERIALS AND METHODS

Bovine heart cardiolipin sodium salt, 1,2-dipalmitoyl-*sn*-glycero-phosphoric acid sodium salt (phosphatidic acid), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol sodium salt (phosphatidylglycerol), 1,2-dipalmitoyl-*sn*-glycerol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS), trimethylchlorosilane, creatine phosphate (CP), creatine phosphokinase (CPK), arachidonic acid (AA), adenosine diphosphate (ADP) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Tris-HCl was from Fluka (Buchs, Switzerland), 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (paf) was a product of Bachem (Bubendorf, Switzerland); and aspirin as a lysine soluble salt (Aspergic) was obtained from Egic Laboratory (Amilly, France). Trimethyl phosphite, purchased from lancaster synthesis Ltd. (East Gate Lancaster, England) was redistilled and kept over molecular sieve (5Å). BN 52021 [3-(1,1-dimethylethyl)hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7a-(epoxymethano)-1H,6aH-cyclopenta[c]furo(2,3b)-[3′2′3,4]cyclopenta[1,2-d]furan-5,9,12(4H)-trione] was a gift from Dr. Braquet (Institute Henri Beaufour, Paris, France); WEB 2086 [3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-2-yl]-1-(4-morpholinyl)-1-propanone] was a gift from Dr. Weber (Boehringer-Ingelheim, Ingelheim, Germany).

Animals. Male New Zealand white rabbits were used as the platelet source.

Buffers and reagents. The following buffers and reagents were used: Tyrode's-gelatin-ethylene-glycoltetraacetic acid (EGTA) buffer solution (Tg-EGTA), pH 6.5 that consisted of KCl, 2.6 mM; $MgCl_2$, 1.0 mM; NaCl,

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Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; BN 52021, 3-(1,1-dimethylethyl)hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7a-(epoxymethano)-1H,6aH-cyclopenta[c]furo(2,3b)-[3′2′3,4]cyclopenta[1,2-d]furan-5,9,12(4H)-trione; BSA, bovine serum albumin; CP, creatine phosphate; CPK, creatine phosphokinase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; IR, infrared; paf, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPP, platelet-poor plasma; PRP, platelet-rich plasma; Tg-EGTA, Tyrode's-gelatin-EGTA buffer; Tg-Ca^{2+} buffer; TLC, thin-layer chromatography; TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; WEB 2086, 3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-2-yl]-1-(4-morpholinyl)-1-propanone.

137 mM; $NaHCO_3$, 12 mM; glucose, 1.0 g/L; EGTA, 0.2 mM; and gelatin, 0.25% (by vol). Tg-Ca^{2+}, pH 7.4, was the Tg-EGTA buffer that contained $CaCl_2$, 12 mM, and Tris-HCl, 10 mM, but no $NaHCO_3$ or EGTA. Ethylenediaminetetraacetic acid (EDTA) solution in saline, 0.2 M, pH 7.2, and ACD solution (citric acid, trisodium citrate and glucose, 0.1 M each), were used as anticoagulants. Paf solutions in BSA saline (2.5 mg/mL) were prepared as previously described (29).

Synthesis and identification of the compounds tested. The structures of cardiolipin, phosphatidic acid, phosphatidylglycerol, and of their analogues are shown in Table 1. The chemical purity of the commercial products (cardiolipin, phosphatidic acid and phosphatidylglycerol) was confirmed by thin-layer chromatography (TLC) on silica gel G plates (0.25-mm thick) using (i) acetone/petroleum ether (1:3, vol/vol) followed by chloroform/methanol/acetic acid/water (80:13:8:0.3, by vol) and (ii) chloroform/methanol/water/ammonium hydroxide (65:35:5:2, by vol) as solvent systems. TLC of the compounds developed either way showed a single fraction with R_f values of 0.36 and 0.46 for cardiolipin, 0.44 and 0.15 for phosphatidic acid and 0.22 and 0.39 for phosphatidylglycerol, respectively, in the two systems. The fatty acid composition of cardiolipin was determined by gas–liquid chromatography. The main constituent fatty acid was linoleic, 87%, and oleic and linolenic acids amounted to 7.2 and 3.1%, respectively. The optical rotation of cardiolipin was $[\alpha]_D^{20} = +5.4°$ (c = 2.55, in chloroform), and the melting point (m.p.) was in the range of 206–208°C.

*1,2-Dipalmitoyloxypropyl-3-(1',2'-dipalmitoyl-*sn*-glycero)phosphate,* [bis*(dipalmitoylglycero)phosphate*]. Dry 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (105 mg, 0.162 mmol) and dry 1,2-dipalmitoyl-*sn*-glycerol (191.6 mg, 0.326 mmol) were dissolved in 1.0 mL anhydrous pyridine, and a solution of TPS (123 mg, 0.492 mmol) in anhydrous pyridine (2.0 mL) was added. The reaction mixture was kept at room temperature for 6–8 h. After completion of the reaction, excess TPS was hydrolyzed by addition of water (1.5 mL), the solvents were evaporated under reduced pressure and the residue was extracted twice with diethyl ether (5 mL).

The product was purified by preparative TLC on silica gel H plates (0.55-mm thick) chloroform/methanol/water (65:25:4, by vol) using as solvent system. The band with R_f 0.72 was scraped off, and the adsorbent was eluted with chloroform/methanol (2:1, vol/vol). The yield of the pure product was 34.5 mg (50.6%) (30).

*1,2-Dipalmitoyloxypropyl-3-(1',2'-dipalmitoyl-*sn*-glycero)phosphonate,* [*phosphono analogue of* bis*(dipalmitoylglycero)phosphate*]. Dry 1,2-dipalmitoyloxypropyl-3-phosphonic acid (300 mg, 0.46 mmol) and 1,2-dipalmitoyl-*sn*-glycerol (547 mg, 0.93 mmol) were dissolved in 2.5 mL of anhydrous pyridine (31,32). A solution of TPS (350 mg, 1.4 mmole) in anhydrous pyridine (4.0 mL) was added to this, and the reaction mixture was kept at room temperature for 8 h. Then water was added (3.0 mL), and the solvents were evaporated under reduced pressure. The residue was extracted twice with diethyl ether (10 mL), and chromatographically pure *bis*(1,2-dipalmitoyl-*sn*-glycero)phosphonate was obtained by preparative TLC on silica gel H plates (0.75-mm thick). The band with an R_f of 0.72 was scraped off the plate, and the adsorbent was eluted with chloroform/methanol (2:1, vol/vol). The yield was 334.9 mg (59.7%), and the m.p. was 59.5°C.

Elemental analysis. Found: C, 69.98%; H, 11.81%; O, 14.98%; P, 2.64%. Calculated: C, 71.06%; H, 11.42%; O, 14.90%; P, 2.62%.

Phosphorus determinations confirmed that the compound was a phosphonolipid because it did not release inorganic phosphorus when subjected to hydrolysis with 6N HCl for 48 h at 110°C. Infrared (IR) spectrum: carbonyl of ester, C=O, at 1,740 cm^{-1}; P=O, at 1,320 and 1,245 cm^{-1}; O=P-OH, at about 2,300 cm^{-1}; P-C, at 1,000, 1,040 and 1,180 cm^{-1}; and C-P stretching at 725 cm^{-1}. Optical rotation $[\alpha]_D^{24} = +7.2°$ (c = 3.6, in benzene).

*1,2-Dipalmitoyloxypropyl-3-(1',2'-diacyl-*sn*-glycero-3-phosphoryl-1,3-*sn*-glycero)phosphonate* [*phosphono analogue of cardiolipin*]. Phosphatidylglycerol (100 mg) was mixed with 1,2-dipalmitoyloxypropyl-3-phosphonic acid (500 mg, 0.077 mmol) in 5.0 mL of anhydrous pyridine (31,32). Then, 230 mg (0.90 mmol) of TPS in 5.0 mL of pyridine was added, and the reaction mixture was kept at room temperature for 8 h. After the completion of the reaction, 2 mL of water was added and the product was extracted with chloroform. The product was purified by preparative TLC on silica gel H plates with chloroform/methanol/water (65:25:4, by vol) as solvent system (R_f 0.96). The yield of the pure product was 63 mg (60%), and the m.p. was 174–175°C.

Elemental analysis. Found: C, 64.90%; H, 10.10%; P, 4.22%. Calculated: C, 65.80%; H, 10.40%; P, 4.40%. The IR spectrum was similar to that reported previously (33). The optical rotation of the compound was $[\alpha]_D^{24} = +6.4°$ (c = 2.4, in chloroform).

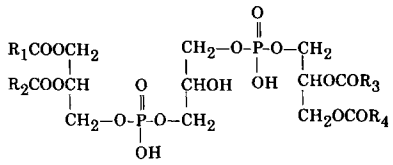
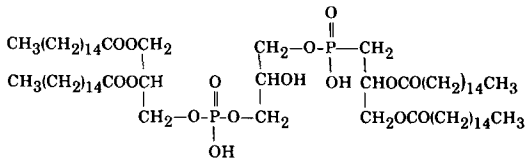
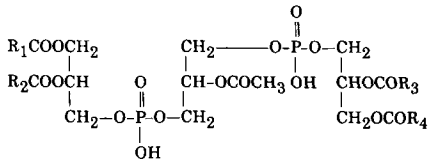
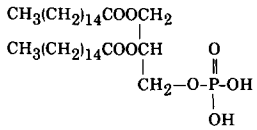
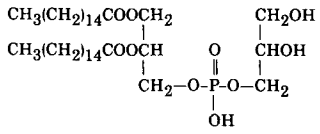
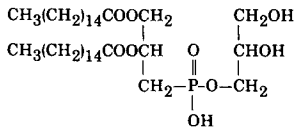
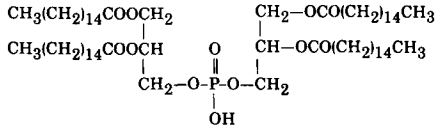
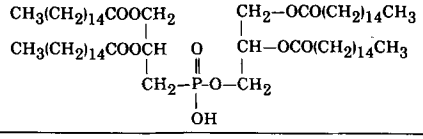
1,2-Dipalmitoyloxypropyl-3-(glycerol-1)phosphonate [*phosphono analogue of phosphatidylglycerol*]. Trimethylchlorosilane (7 mg, 0.065 mmol) was mixed with LiBr (5 mg, 0.052 mmol) in 3.0 mL of CH_3CN. A solution of phosphatidylglycerol (20 mg, 0.026 mmol) in 2.0 mL of CH_3CN was added to this, and the reaction was completed within 20 h. The reaction mixture was taken up in diethyl ether (40 mL) and washed successively with H_2O, then with $NaHCO_3$ solution (10% wt/vol), and finally with NaCl solution (5% wt/vol), and the ether layer was dried over anhydrous Na_2SO_4. Evaporation of the solvent afforded the bromohydrin. The dry bromohydrin was reacted at 120°C for 24 h with trimethylphosphite according to the procedure of Kosolapoff (33). The crude product was hydrolyzed with trimethylchlorosilane/NaI in CH_3CN at room temperature for 15 min, and the final product was purified by preparative TLC on silica gel G plates (0.75-mm thick) using chloroform/methanol/water (65:25:4, by vol). The R_f value was 0.57, and the optical rotation of the compound was $[\alpha]_D^{24} = +1.8°$ (c = 9, in chloroform). The m.p. was 57–59°C (uncorrected), and the IR spectrum in the region 900–1,400 cm^{-1} was similar to that reported previously (31,32).

Acetyl-cardiolipin. Bovine heart cardiolipin was acetylated with acetic anhydride at 65°C for 3 h. The product was purified by preparative TLC on silica gel plates (0.55-mm thick) using the solvent system chloroform/methanol/water (80:20:2, by vol). The R_f of acetyl-cardiolipin was 0.8, and the R_f of cardiolipin was 0.72.

Platelet-rich plasma (PRP) preparation. Whole blood from rabbits was mixed with ACD (9:1, vol/vol) in polyethylene tubes and centrifuged at 375 × *g* for 20 min at

TABLE 1

Structure and Biological Activity of Cardiolipin, Phosphatidic Acid, Phosphatidylglycerol and Some Structural Analogues

No.	Name/activity	Structure
1	Cardiolipin/inhibition	
2	Phosphonocardiolipin/inhibition	
3	Acetyl-cardiolipin/aggregation	
4	Phosphatidic acid/inactive	
5	Phosphatidylglycerol/inactive	
6	Phosphonophosphatidylglycerol/inactive	
7	*bis*(Dipalmitoylglycero)phosphate/inactive	
8	Phosphono analogue of 7/inactive	

room temperature. The PRP was collected and centrifuged at 1,400 × *g* for 15 min at room temperature to obtain platelet-poor plasma (PPP). Platelets were counted on a hematocytometer according to the method of Brecher and Cronkite (34), and PRP was diluted with homologous PPP to yield a concentration of 3.0 ± 0.2 × 10^8 cells/mL.

Washed rabbit platelet preparation. Platelets were prepared from whole blood (collected in EDTA, 1:40, vol/vol) by successive centrifugations and dilutions in Tg-EGTA buffer according to the method of Ardlie *et al.* (35) as modified by Benveniste *et al.* (29). The washed platelets were suspended in an appropriate volume of Tg-EGTA to give a concentration of 2.5 × 10^9 cells/mL.

Platelet aggregometry. Platelet aggregation was monitored with a single channel aggregometer (ChronoLog Corp., Havertown, PA) under constant sitrring (1,200 rpm at 37°C). PRP was treated with aspirin (1 mM), and with CP (5 mM) and CPK (40 U/mL), for 15 min and 1 min, respectively, before aggregation was measured. Cardiolipin solutions in ethanol/water (60%, vol/vol) were added to 500 μL of PRP, in volumes of 1-5 μL. In inhibition studies, PRP was incubated with cardiolipin for 30 s and 10 min before the paf addition. Aggregation measurements with washed rabbit platelets were done as follows: 100 μL of platelet suspension pre-incubated with aspirin (1 mM) for 15 min was transferred into the cuvette of the aggregometer in which 400 μL of Tg-Ca^{2+} buffer containing CP/CPK (5 mM/40 U/mL, respectively) was added. Antagonist solutions in ethanol/water (60%, vol/vol), in volumes of 1-5 μL were added to paf, 30 s before the platelet suspension. In other experiments, the platelet suspension was incubated in the cuvette with the antagonist for 10 min prior to paf addition. For the aggregation studies with ADP or AA, CP/CPK or aspirin (respectively) was omitted. Inhibition studies with the specific paf inhibitors, BN 52021 (solubilized in dimethylsulfoxide) and WEB 2086 (solubilized in Sorenson's phosphate buffered saline, pH 7.4) were performed as previously described (36,37).

RESULTS

General comments. The structures of the compounds tested as possible paf inhibitors as well as their biological activities are shown in Table 1. The compounds were chosen with the aim of elucidating the possible relationship between cardiolipin structure and biological activity.

Experiments with washed rabbit platelets. The EC_{50} value for paf (dose for half-maximal aggregation) was 1 × 10^{-10} M, while at a dose of 2 × 10^{-10} M paf caused submaximal aggregation. The possible inhibitory effect of cardiolipin and of its analogues and derivatives was tested at the above paf concentrations. As shown in Figure 1, cardiolipin inhibited platelet aggregation induced by paf at 1 × 10^{-10} M and 2 × 10^{-10} M in a concentration-dependent manner with an IC_{50} value of 8.4 ± 0.8 × 10^{-7} M and 2.6 ± 0.6 × 10^{-6} M, respectively. In contrast, cardiolipin in concentrations ranging from 1 × 10^{-7} to 1 × 10^{-5} either failed to inhibit or only slightly inhibited platelet aggregation induced by 0.2-1.0 μM AA or 0.9-1.8 μM ADP. Under the same experimental conditions, the phosphono analogue of cardiolipin showed a more potent inhibitory effect than cardiolipin itself against the aggregation induced by paf (1 × 10^{-10} M); the IC_{50} value was 3 × 10^{-7} M. Also, phosphonocardio-

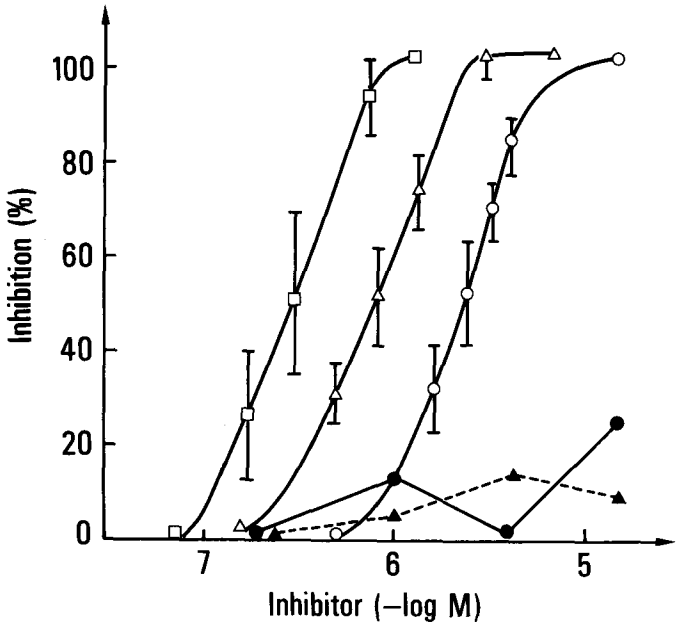


FIG. 1. The inhibitory effects of cardiolipin and phosphonocardiolipin on platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (paf)-, arachidonic acid (AA)- and adenosine diphosphate (ADP)-induced washed rabbit platelet aggregation. ○, Cardiolipin against 2×10^{-10} M paf; △, cardiolipin against 1×10^{-10} M paf; ●, cardiolipin against 1 μM AA; ▲, cardiolipin against 0.9 μ M ADP; and □, phosphonocardiolipin against 1×10^{-10} M paf. Each point represents the mean ± 1 SD of ten experiments.

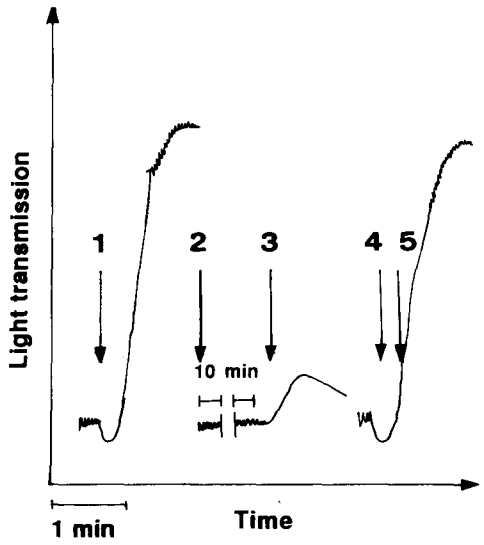


FIG. 2. Representative tracings of the inhibitory effect of cardiolipin on washed rabbit platelets. 2×10^{-10} M (1); 7×10^{-6} M cardiolipin (2); 2×10^{-10} M paf (3); 2×10^{-10} M paf (4); 7×10^{-6} M cardiolipin (5). Abbreviation as in Figure 1.

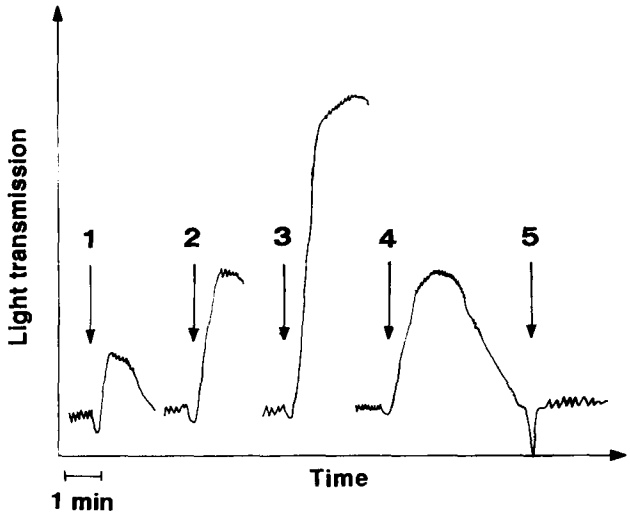


FIG. 3. Representative tracings of the aggregatory effect of acetyl-cardiolipin. 8×10^{-6} M acetyl-cardiolipin (1); 1.5×10^{-5} M acetyl-cardiolipin (2); 2×10^{-10} M paf (3); 1.5×10^{-5} M acetyl-cardiolipin (4); 2×10^{-10} M paf (5). Abbreviation as in Figure 1.

lipin in concentrations up to 1×10^{-5} M failed to inhibit platelet aggregation induced by AA (0.2-1.0 μM) or ADP (0.9-1.8 μM) (Fig. 1). Phosphatidic acid, phosphatidylglycerol, *bis*(dipalmitoylglycero)phosphate and their phosphono analogues at concentrations up to 1×10^{-5} M were ineffective in inhibiting platelet aggregation induced by paf, AA or ADP (results not shown). Similar results were obtained when PC and PE were tested as possible paf inhibitors at the above concentrations. In some experiments, platelets were preincubated with cardiolipin for 10 min prior to paf addition. As shown in Figure 2, the inhibitory effect of cardiolipin was preserved even after a 10-min incubation with platelets. In contrast, cardiolipin added to the platelets after paf failed to reverse the aggregation (Fig. 2).

In contrast to other compounds tested, which failed to induce platelet aggregation or lysis even at concentrations up to 1×10^{-5} M, acetyl-cardiolipin at concentrations higher than 1×10^{-6} M exerted a weak aggregatory effect on washed rabbit platelets that reached a maximum (%) at a concentration of 1.7×10^{-5} M (Fig. 3). This maximal aggregation was totally inhibited by 1×10^{-5} M BN 52021 or 1×10^{-6} M WEB 2086. Also, platelets treated with acetyl-cardiolipin were insensitive to the aggregatory effect of paf (Fig. 3).

Experiments with rabbit PRP. The purpose of these experiments was to study the effect of plasma on the activity of cardiolipin as a paf inhibitor. The inhibitory effect of cardiolipin was examined in respect to platelet aggregation induced by paf, 6×10^{-8} M, which resulted in a submaximal response. As shown in Figures 4 and 5, cardiolipin added 30 s prior to paf addition inhibited platelet aggregation in a dose-dependent manner up to a concentration of $1.5 \pm 0.3 \times 10^{-5}$ M, at which 50% inhibition was observed. This inhibitory effect decreased with higher cardiolipin concentrations, and was completely lost at a concentration of $1 \pm 0.5 \times 10^{-4}$ M. Incubation of PRP with cardiolipin at 1.5×10^{-5} M for 10 min at room temperature caused a complete loss of inhibition. Finally, acetyl-cardiolipin at concentrations higher than 1×10^{-5} M exhibited a weak aggregatory effect (Fig. 5).

DISCUSSION

The existence of endogenous paf inhibitors in various mammalian cells and tissues has been described previously (26-28), but the chemical structures of these inhibitors are still unknown. Also, little is known about their

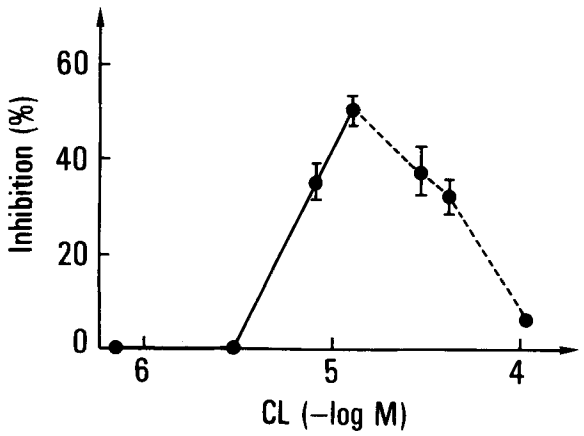


FIG. 4. The inhibitory effect of cardiolipin (CL) on rabbit platelet-rich plasma aggregation induced by 6×10^{-8} M paf. Each point is the mean ± 1 SD of four experiments. Abbreviation as in Figure 1.

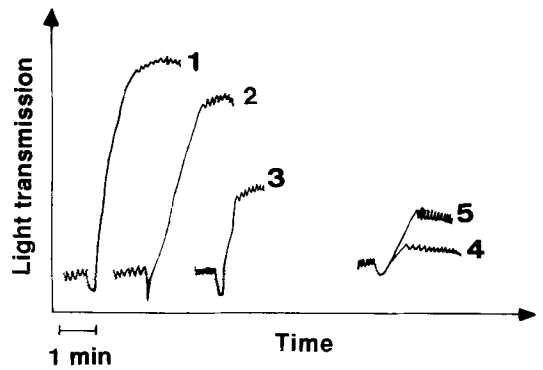


FIG. 5. Representative tracings of the effects of cardiolipins on rabbit platelet-rich plasma. The inhibitory effect of cardiolipin at 1.5×10^{-5} M (3) and 0.9×10^{-5} M (2) on platelet aggregation induced by 6×10^{-8} M paf (1). The aggregatory effect of acetyl-cardiolipin at 1.5×10^{-5} (4) and 5×10^{-5} M (5). Abbreviation as in Figure 1.

biological role as anti-paf agents under normal conditions or in various diseases in which paf is thought to be involved. In the present study we demonstrated that cardiolipin, a phospholipid that is a normal constituent of mammalian tissues, specifically inhibits rabbit platelet aggregation induced by paf *in vitro*.

Cardiolipin can constitute 2-10% of the total phospholipids of various mammalian tissues and is characteristically associated with subcellular membranous particles, particularly mitochondria; it is thought to play an important role as a structural component in the respiratory chain (38). As far as we are aware, little research has been done on the relationship between cardiolipin and platelets, although cardiolipin is a minor constituent of platelet phospholipids (39). In high concentrations (10-100-fold greater than those employed in this study), cardiolipin exhibits a significant Ca^{2+} ionophoretic activity on washed human platelets and also enhances platelet aggregation caused by ADP and epinephrine in human PRP (40). Clinical interest in the possible involvement of anti-cardiolipin antibodies in thrombocytopenia observed in Lupus erythematosus and related auto-immune disorders has been increasing, although little is known about the mode of action (41,42).

Our results show that cardiolipin is a specific inhibitor of paf in washed rabbit platelet aggregation *in vitro* because it failed to inhibit the aggregation induced by AA and ADP. Of particular interest is the observation that cardiolipin exhibits its inhibitory effect at concentrations similar to those of known, specific paf antagonists (25). These concentrations were lower than those reported for other endogenous paf inhibitors (28) and lower than the concentrations at which various phospholipids cause nonspecific paf inhibition, possibly due to general disturbance of the platelet membrane structure (43). It is unknown whether or not within our range of concentrations, and under our experimental conditions, cardiolipin forms micelles with paf which could explain inhibition. Nevertheless, when cardiolipin is incubated with washed rabbit platelets 10 min prior to paf addition, it preserves its inhibitory effect. A decrease in cardiolipin inhibitory effect would be expected if paf molecules were sequestered in micelles under our experimental conditions. Our study also demonstrates that the cardiolipin molecule must be intact to exhibit its specific anti-paf action. Any alteration of the structure of the molecule results in a complete loss of observed biological activity; also, lyso-monocardiolipin has been reported to be inactive (27).

Of particular interest is the observation that acetyl-cardiolipin exhibits a paf-like aggregatory effect on platelets because it aggregates platelets in the presence of aspirin and CP/CPK, while its aggregatory effect is completely inhibited by the paf receptor antagonists, BN 52021 and WEB 2086. Also, platelets treated with acetyl-cardiolipin are desensitized to the aggregatory effect of paf. This observation supports the hypothesis that the acetyl-cardiolipin aggregatory effect and the cardiolipin inhibitory effect on washed rabbit platelets are paf receptor-mediated phenomena, although both compounds are not structural analogues of paf.

The complicated behavior of cardiolipin in PRP is possibly due to its binding to various plasma constituents, such as enzymes, proteins and ions leading to configurational changes (38). These interactions may be responsible for the loss of biological activity, particularly at high cardiolipin concentrations or after a prolonged incubation with PRP.

Our study has provided evidence that cardiolipin is a specific paf inhibitor. Cardiolipin may play an anti-paf role, *in vivo*, in cell-to-cell interactions in which paf is involved. In plasma and in other biological fluids, it may have a special pathophysiological significance through its anti-paf action.

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Synthesis of Deuterated Cyclopropene Fatty Esters Structurally Related to Palmitic and Myristic Acids

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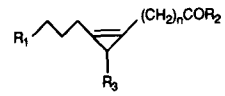
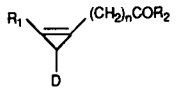
To develop a synthesis of tritiated cyclopropene fatty acids (CPFA), compounds that should prove useful for affinity labeling of desaturases in insect pheromone biosynthetic studies, a series of novel, selectively deuterated CPFA analogues was prepared and characterized. In methyl [16-^{2}H]12,13-methylene-12-hexadecenoate, the incorporation of deuterium was achieved by treatment of the corresponding ω-chloro derivative with sodium borodeuteride in dimethylsulfoxide at 70°C for 24 h (67% yield) following conventional procedures. Alkylation of the tetrahydropyranyl derivative of 13-tridecynol in the presence of lithium diisopropylamide in tetrahydrofuran at −20°C with 1-chloro-3-iodopropane in hexamethylphosphoramide, followed by Jones oxidation of the crude product, yielded 16-chloro-12-hexadecynoic acid (54%), which was esterified to the corresponding methyl ester by treatment with potassium carbonate and methyl iodide in dimethylformamide. Treatment of this acetylenic ester with ethyldiazoacetate in the presence of activated copper-bronze as catalyst followed by hydrolysis in KOH solution at room temperature yielded 16-chloro-12,13-(carboxymethylene)-12-hexadecenoic acid. This diacid was treated with excess oxalyl chloride to give the corresponding diacyl chloride, which was decarbonylated in a diethyl ether solution with zinc chloride, and the cyclopropenium ions thus formed were added at −40°C to a methanolic sodium hydroxide solution of sodium borohydride to give methyl 16-chloro-12,13-methylene-12-hexadecenoate. Analogous procedures were followed to prepare methyl [17-^{2}H]10,11-methylene-10-hexadecenoate, methyl [17-^{2}H]11,12-methylene-11-hexadecenoate and methyl [17-^{2}H]12,13-methylene-12-hexadecenoate from the corresponding diacids using sodium borodeuteride in the reduction of the cyclopropenium ions. Alternatively, methyl [2,2,3,3-$^2H_4$]hexadecynoate, prepared by reaction of methyl 2,11-hexadecadiynoate with magnesium in deuterated methanol at room temperature, was submitted to the above cyclopropenylation and reductive decarbonylation sequence to give methyl [2,2,3,3,17-$^2H_5$]-11,12-methylene-11-hexadecenoate. In summary, complementary methods for the selective incorporation of one to five deuterium atoms into cyclopropene fatty acids, at different sites, in moderate to high yields have been developed. The methods should easily be applicable to the preparation of the corresponding tritiated analogues.
Lipids 28, 1125–1130 (1993).

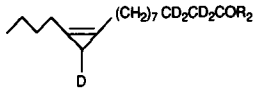
Desaturases are key enzymes in the biosynthesis of lepidopteran sex pheromones (1). Our interest in these unique enzymes led us to the search for specific and irreversible desaturase inhibitors that would be useful for affinity labeling. We had previously found (2,3) that certain cyclopropene fatty acids (CPFA) are potent and presumably irreversible inhibitors of *Z*(11) and *Z*(9) desaturation of palmitic and *E*(11)-tetradecenoic acids, respectively, in *Spodoptera littoralis* pheromone glands. We thought that these compounds, conveniently radiolabeled, might be suitable affinity labels for these enzymes. Additional bioassays indicated that compounds **1b**, **3b**, **4b** and **5b** would label both the *Z*(11) and the *Z*(9) desaturase, whereas **2b** and **6b** would be specific for the *Z*(9) and the *Z*(11) enzymes, respectively. As a pilot study to the synthesis of tritiated cyclopropene fatty acids, we describe in this paper the preparation of the deuterated cyclopropene fatty esters **1a**–**6a**. In most cases, deuterium was introduced in the last step of the synthesis to optimize the procedures for the preparation of the corresponding radioactive materials.



1a: R_1=D, R_2=OCH_3, R_3=H, n=10
1b: R_1=D, R_2=OH, R_3=H, n=10
2a: R_1=H, R_2=OCH_3, R_3=D, n=8
2b: R_1=H, R_2=OH, R_3=D, n=8



3a: R_1=C_3H_7, R_2=OCH_3, n=10
3b: R_1=C_3H_7, R_2=OH, n=10
4a: R_1=C_4H_9, R_2=OCH_3, n=9
4b: R_1=C_4H_9, R_2=OH, n=9
5a: R_1=C_5H_{11}, R_2=OCH_3, n=8
5b: R_1=C_5H_{11}, R_2=OH, n=8



6a: R=OCH_3
6b: R=OH

RESULTS AND DISCUSSION

Cyclopropene fatty ester **1a**, in which deuterium is placed at the ω-position, was prepared as illustrated in Scheme 1. A chlorine atom was selected as precursor of deuterium. Alkylation of **7a** with 1-chloro-3-iodopropane, followed by Jones oxidation and methylation of the resulting fatty acid **8b**, afforded **8a** in 45% overall yield. The cyclopropene ring in **10b** was formed by reaction of alkyne **8a** with ethyldiazoacetate in the presence of activated Cu-bronze as catalyst (4), and the resulting diester **10a** was hydrolyzed at room temperature. Decarbonylation of diacyl

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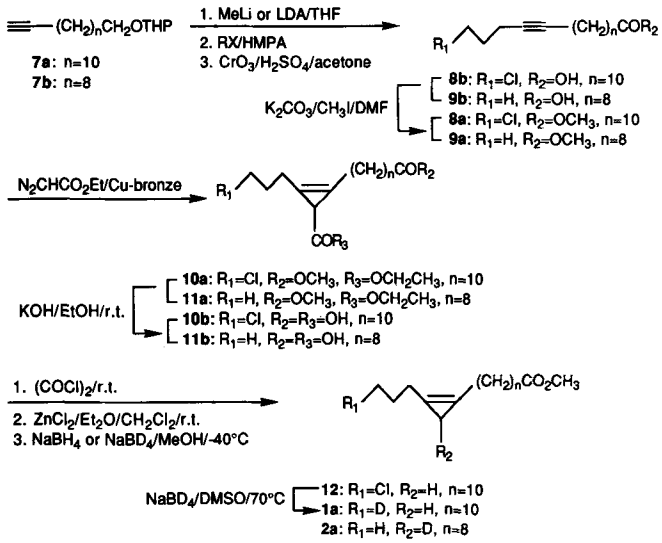
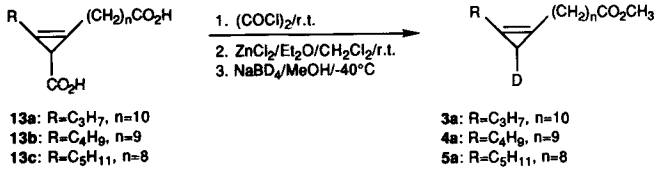
Abbreviations: CPFA, cyclopropene fatty acids; DEPT, distortionless enhancement by polarization transfer; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DQ-COSY, double quantum filtered correlation spectroscopy; IR, infrared; HETCOR, heteronuclear correlation; HMPA, hexamethylphosphoric acid triamide; HRMS, high-resolution mass spectrometry; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMEDA, tetramethylethylenediamine.



SCHEME 1



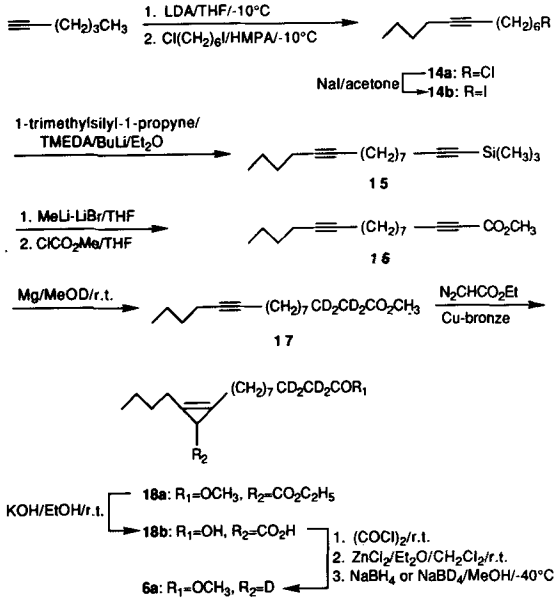
SCHEME 2

chloride of **10b** was accomplished with $ZnCl_2$. Although solid $ZnCl_2$ had been used in previous studies (4,5), we found that the reaction proceeded more rapidly when $ZnCl_2$ was used as diethyl ether solution, which is commercially available. Reduction of the cyclopropenium ions thus formed was accomplished with $NaBH_4$ in NaOH/MeOH at low temperature. Although a molar cation/hydride ratio of 1:4 was used in previous syntheses (4,5), we found that the ratio could be lowered to 1:2 without significant decrease in yield. This modification was particularly important in the case of the ring-deuterated compounds as it allowed less labeling reagent to be used. The chlorine atom remained unaffected under these reduction conditions, but chlorine was successfully reduced by $NaBD_4$ in dimethylsulfoxide (DMSO) at 70°C (6). It should be noted that in the ^1H nuclear magnetic resonance (NMR) spectra of both diester **10a** and diacid **10b**, signals corresponding to the methylenes C-11, C-14 and C-16 revealed the diastereotopic nature of the protons at these positions. Assignment of ^{13}C signals in the different cyclopropenes was carried out on the basis of distortionless enhancement by polarization transfer (DEPT), heteronuclear correlation (HECTOR) and double quantum filtered correlation spectroscopy (DQ-COSY) experiments, with some compounds.

Cyclopropene fatty ester **2a** was prepared as shown in Scheme 1. Alkylation of acetylene **7b** with 1-bromopropane, followed by Jones oxidation and methylation of acid **9b** produced **9a**. Transformation of **9a** into the final product was accomplished as described above for the C_{16} compounds.

The synthesis of ring-labeled cyclopropene fatty esters **3a–5a** (Scheme 2) was accomplished by decarbonylation, followed by reduction of diacyl chlorides of **13a–c**, which were prepared as previously described (5). Both decarbonylation and cyclopropenium ion reduction were carried out as previously indicated, although $NaBD_4$ was used as reducing agent.

The pentadeuterated cyclopropene **6a** was also prepared. Additional deuterium atoms were introduced at $\alpha,\alpha,\beta,\beta$-positions to avoid chain-shortening of the inhibitor in the gland and thus increase its specificity toward the *Z*(11) desaturase (3). The synthesis of **6a** was carried out as shown in Scheme 3. Alkylation of 1-hexynyllithium with 1-chloro-6-iodohexane yielded the chloroalkyne **14a**, which was transformed into **14b** by treatment with NaI in acetone. Propargylation was achieved in high yields by alkylation of **14b** with lithio-1-trimethylsilylpropyne (7,8) which, in turn, was readily obtained by reaction of 1-trimethylsilyl-1-propyne with tetramethylethylenediamine and an equivalent amount of *n*-butyllithium (8). The presence of a trimethylsilyl group was very convenient as it replaced the acidic acetylenic proton in propyne, allowing metallation of the methyl group for the propargylation reaction to take place. Metallation at C-1 in the resulting diyne **15** was achieved by direct treatment with MeLi-LiBr complex (9). Quenching of the acetylide thus formed with methyl chloroformate afforded the expected ester **16**. Deuterium atoms were introduced by reduction with Mg/CH_3OD (10). This reaction proceeded by selective reduction of the conjugated triple bond at C-2, leaving the isolated triple bond at C-11 intact. Transformation of tetradeuterated ester **17** into the cyclopropene **6a** was accomplished as indicated, following the general sequence of reactions.



SCHEME 3

EXPERIMENTAL PROCEDURES

Commercial-grade reagents and solvents were directly used as supplied, with the following exceptions: Diethyl ether and tetrahydrofuran (THF) (SDS, Barberá del Vallés, Spain) were distilled over Na/benzophenone under N_2, diisopropylamine (Fluka, Buchs, Switzerland) over KOH pellets and CH_2Cl_2 (SDS) over P_2O_5. Dimethylformamide (DMF) (Panreac, Montcada i Reixac, Spain), hexamethylphosphoramide (HMPA) (Fluka) and tetramethylethylenediamine (TMEDA) (Fluka) were distilled and kept over molecular sieves (3Å). Reactions sensitive to oxygen and moisture were carried out under an argon atmosphere. Purification of products by column chromatography was done using Merck (Darmstadt, Germany) 70-230 mesh silica gel deactivated with 10% water. Thin-layer chromatography (TLC) was carried out using Merck $60F_{254}$ (0.25-mm) sheets. Products were generally isolated by pouring the reaction mixture onto ice, and by extracting with solvent, washing with brine and drying over $MgSO_4$. Elemental analyses were performed on a Carlo Erba (Milano, Italy) elemental analyzer model 1106. Fourier transform infrared spectra were recorded on films using a Michelson Bomem (Montreal, Quebec, Canada) MB-120 spectrometer. ^1H and ^{13}C NMR spectra were obtained on $CDCl_3$ solutions with either a Bruker (Karlsruhe, Germany) WP80SY, a Varian (Palo Alto, CA) XL200 or a Varian Unity-300 spectrometer at 80, 200 or 300 MHz, respectively, for ^1H, and at 20, 50 or 75 MHz for ^{13}C. Low-resolution mass spectra were measured on a Hewlett-Packard (Palo Alto, CA) HP 5995 mass spectrometer coupled to a gas chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm i.d.) SPB-5 from Supelco (Bellefonte, PA). High-resolution mass spectra (HRMS) were measured on an Autospec-Q (VG Analytical, Fisons, Rodano, Italy) mass spectrometer.

10-Tetradecynoic acid (**9b**) *(Ref. 11).* Under argon, 4.8 mL (34 mmol) of diisopropylamine in 34 mL of dry THF was placed in a three-neck, round-bottom flask. The solution was stirred at −10°C, and 29 mL (35 mmol) of a 1.2 M solution of BuLi in hexane was added. The mixture was stirred for 2 h at 0°C, and then 7.8 g (31 mmol) of **7b** in 89 mL of THF was added at the same temperature. Stirring was continued for 1 h, and then the acetylide was treated with a solution of 3.5 mL (35 mmol) of 3-bromopropane in 80 mL of HMPA. After stirring for a further 18 h, 8.1 g of an oil was isolated using hexane for extraction. Jones oxidation of 0.706 g of the oil under standard conditions (5) afforded **9b** in 78% yield from **7b** after column chromatography using CH_2Cl_2/MeOH (97:3, vol/vol) as eluent. Infrared (IR): 3550-2500, 2930, 2860, 1710, 1460 cm^{-1}. ^1H NMR (80 MHz); δ 9.75 (*s*, 1H, COOH), 2.13 (*c*, 6H, CH_2CO and $CH_2C{\equiv}$), 1.10-1.60 (*b*, 14H, $\equiv CC(CH_2)_6$ and CH_2CH_3), 0.89 (*t*, J = 7.0 Hz, 3H, CH_3).

16-Chloro-12-hexadecynoic acid (**8b**). The procedure for the synthesis of **9b** was applied to the alkylation of **7a** (1.2 g, 4.25 mmol). Jones oxidation of the crude tetrahydropyranyl derivative (1.0 g, 2.8 mmol) afforded **8b** in 54% yield after purification by column chromatography and elution with CH_2Cl_2/MeOH (97:3, vol/vol). Anal: Calcd. for $C_{16}H_{27}O_2Cl$: C, 67.01; H, 9.42. Found: C, 67.08; H, 9.36. IR: 3500-2500, 2927, 2854, 1708, 1288 cm^{-1}. ^1H NMR (200 MHz): δ 3.64 (*t*, J = 6.4 Hz, 2H, CH_2Cl), 2.38-2.28 (*c*, 4H, CH_2CO and $ClCCCH_2C{\equiv}$), 2.12 (*tt*, J = 6.8 and 2.4 Hz, 2H, $CCCCH_2C{\equiv}$), 1.92 (*m*, J = 6.4 Hz, 2H, $ClCCH_2$), 1.62 (*c*), 1.42 (*c*) and 1.27 (*b*, 16H, $\equiv CC(CH_2)_8$). ^{13}C NMR (50 MHz): 180.23 (C-1), 81.41 (C-12), 77.99 (C-13), 43.78 (C-16), 34.06 (C-2), 31.79 (C-15), 29.42, 29.34, 29.19, 29.08, 29.03 and 28.81 (C-4 to C-10), 24.64 (C-3), 18.68 (C-11), 16.20 (C-14).

Methyl 16-chloro-12-hexadecynoate (**8a**). Esterification of **8b** (0.65 g, 2 mmol) with K_2CO_3 (1.1 g, 7.9 mmol) and CH_3I (0.5 mL, 8 mmol) in DMF (10 mL) afforded **8a** in 95% yield after column chromatography and elution with hexane/diethyl ether (10:1, vol/vol). Anal: Calcd. for $C_{17}H_{29}ClO_2$: C, 67.89; H, 9.65. Found: C, 67.93; H, 9.71. IR: 2927, 2854, 1739, 1434, 1170 cm^{-1}. ^1H NMR (200 MHz): δ 3.65 (*s*, 3H, OCH_3), 3.64 (*t*, J = 6.4 Hz, 2H, CH_2Cl), 2.38-2.25 (*c*, 4H, CH_2CO and $ClCCCH_2C{\equiv}$), 2.12 (*tt*, J = 6.8 and 2.4 Hz, 2H, $CCCCH_2C{\equiv}$), 1.91 (*m*, J = 6.6 Hz, 2H, $ClCCH_2$), 1.60 (*c*), 1.42 (*c*) and 1.27 (*b*, 16H, $\equiv CC(CH_2)_8$). ^{13}C NMR (50 MHz): 174.26 (C-1), 81.39 (C-12), 77.95 (C-13), 51.39 (OCH_3), 43.76 (C-16), 34.06 (C-1), 31.77 (C-15), 29.41, 29.34, 29.19, 29.10, 29.06, 28.97, 28.80 (C-4 to C-10), 24.90 (C-3), 18.65 (C-11), 16.18 (C-14). MS: *m/z* 300 (5%), 130 (50%), 116 (51%), 81 (100%).

Methyl 10-tetradecynoate (**9a**). The same esterification and purification procedures applied to **8b** (0.380 *g*, 1.60 mmol) furnished **9a** in 70% yield. Anal: Calcd. for $C_{15}H_{26}O_2$: C, 75.63; H, 10.92. Found: C, 75.59; H, 10.88. IR: 2930, 2860, 1750, 1465, 1440, 1170 cm^{-1}. ^1H NMR (80 MHz): δ 3.66 (*s* 3H CH_3O), 2.12 (*c*, 6H, CH_2CO and $CH_2C{\equiv}$), 1.20-1.80 (*b*, 14H, $\equiv CC(CH_2)_6$ and CH_2CH_3), 0.86 (*t*, J = 7.4 Hz, 3H, CH_3C).

1-Iodo-7-dodecyne (**14b**). The alkylation procedure described for the synthesis of **9b** applied to 2.5 g (40.3 mmol) of 1-hexyne and 7.8 g (31.7 mmol) of 1-chloro-6-iodohexane afforded 5.8 g of **14a**, which was directly treated with 9 g (60 mmol) of NaI in 30 mL of dry acetone under reflux for 36 h. Extraction with diethyl ether followed by column chromatography and elution with hexane/diethyl ether (5:1, vol/vol) yielded 5 g (17 mmol, 63%) of pure **14b**. Anal: Calcd. for $C_{12}H_{21}I$: C, 49.31; H, 7.19. Found: C, 49.43; H, 7.24. IR: 2956, 2935, 2856, 2219, 1461, 1433, 1197, 727 cm^{-1}. ^1H NMR (200 MHz): δ 3.17 (*t*, J = 7.0 Hz, 2H, CH_2I), 2.13 (*c*, 4H, $CH_2C{\equiv}$), 1.82 (*c*, 2H, $ICCH_2$), 1.40 (*c*, 10H, $\equiv CC(CH_2)_3$ and $(CH_2)_2CH_3$), 0.89 (*t*, J = 7.2 Hz, 3H, CH_3). ^{13}C NMR (50 MHz): δ 80.31 and 79.72 (C-7 and C-8), 33.34 (C-2), 31.17 (C-10), 29.95 (C-3), 28.77 (C-5), 27.59 (C-4), 21.85 (C-11), 18.58 (C-9), 18.36 (C-6), 13.57 (C-12) and 6.95 (C-1).

1-Trimethylsilyl-1,10-pentadecadiyne (**15**). Into a three-neck, round-bottomed flask was placed (under argon) 1.5 mL (10 mmol) of dry TMEDA, and 6.4 mL (10 mmol) of a 1.6 M solution of BuLi was slowly added at −5°C. After 2.5 h of stirring, 1.5 mL (10 mmol) of 1-trimethylsilyl-1-propyne in 10 mL of dry diethyl ether was added. This mixture was stirred at −5°C for 30 min and was reacted with 3 g (10 mmol) of iodoalkyne **14b** in 10 mL of dry diethyl ether. The ice bath was removed, and the mixture stirred at room temperature for 12 h. Extraction with hexane afforded 2.6 g (9.5 mmol, 92%) of diyne **15**. A small sample was purified by bulb-to-bulb distillation (170°C/1 Torr) for characterization. Anal: Calcd. for $C_{18}H_{32}Si$: C, 78.26; H, 11.59. Found: C, 78.15; H, 11.43. IR: 2483, 2433, 2089, 1933, 1464, 1245, 845 cm^{-1}. ^1H NMR (200 MHz): δ 2.21 (*t*, J = 7.2 Hz, 2H, $CH_2C{\equiv}CSi$), 2.14 (*t*, J = 6.6 Hz,

4H, $CH_2C{\equiv}CC$), 1.42 (*c*, 14H, $\equiv CC(CH_2)_5$ and $(CH_2)_2CH_3$), 0.90 (*t*, J = 7.4 Hz, 3H, CH_3C), 0.14 (*s*, 9H, CH_3Si). ^{13}C NMR (20 MHz): δ 107.47 (C-2), 84.15 (C-1), 79.97 (C-10 and C-11), 31.26 (C-13), 29.02 and 28.59 (C-4 to C-8), 21.87 (C-14), 19.75 (C-3), 18.66 and 18.35 (C-9 and C-12), 13.57 (C-15), 0.12 (CSi). MS: *m/z* 203 (10%), 73 (100%).

Methyl 2,11-hexadecadiynoate (**16**). A solution of 0.5 g (1.8 mmol) of **15** in 10 mL of THF was treated at 0°C with 1.2 mL (1.8 mmol) of a 1.5 M solution of MeLi/LiBr in diethyl ether, and the mixture was stirred for 3 h. The resulting acetylide was transferred to a solution of methyl chloroformate (0.155 mL, 2 mmol) in 3 mL of THF at −30°C. Stirring was continued for 40 min, and the reaction mixture was treated with a saturated aqueous solution of NH_4Cl and extracted with diethyl ether, affording 350 mg of an oil that was purified by column chromatography using hexane/diethyl ether (5:1, vol/vol) to obtain 230 mg (0.88 mmol, 49%) of pure **16**. Anal: Calcd. for $C_{17}H_{26}O_2$: C, 77.86; H, 9.92. Found: C, 77.94; H, 10.03. IR: 2931, 2858, 1749, 1718, 1434, 1253 cm^{-1}. ^1H NMR (200 MHz): δ 3.74 (*s*, 3H, CH_3O), 2.31 (*t*, J = 7.0 Hz, 2H, $CH_2C{\equiv}CCO$), 2.12 (*c*, 4H, $CCH_2C{\equiv}CCH_2$), 1.40 (*c*, 14H, $\equiv CC(CH_2)_5$ and (CH_2CH_3), 0.88 (*t*, J = 7.0 Hz, 3H, CH_3C). ^{13}C NMR: δ 154.11 (C-1), 89.81 (C-3), 80.24 and 79.93 (C-11 and C-12), 72.80 (C-2), 52.49 (CH_3O), 31.20 (C-14), 28.98, 28.65, 28.50, 27.39 (C-5 to C-9), 21.88 (C-15), 18.64, 18.57 and 18.37 (C-4, C-10 and C-13), 13.58 (C-16). MS: *m/z* 262 (8%), 231 (20%), 205 (25%), 67 (100%).

Methyl [2,2,3,3-$^2H_4$]11-hexadecynoate (**17**). A mixture of ester **16** (0.922 g, 3.52 mmol) and Mg (0.422 g, 17.6 mmol) in 26 mL of CH_3OD was stirred at room temperature for 20 h. After this time, the mixture was poured onto ice and acidified with 0.1 N HCl. Extraction with hexane yielded 0.720 g of a material that was purified by column chromatography and elution with hexane/diethyl ether (5:1, vol/vol) to afford 590 mg (2.2 mmol, 62%) of pure **17**. HRMS: Calcd. for $C_{17}H_{26}D_4O_2$: 270.249687. Found: 270.248833. IR: 2929, 2856, 1739, 1267 cm^{-1}. ^1H NMR: δ 3.65 (*s*, 3H, CH_3O), 2.12 (*c*, 4H, $CH_2C{\equiv}$), 1.47-1.27 (*c*, 16H, $\equiv CC(CH_2)_6$ and $(CH_2)_2CH_3$), 0.87 (*t*, J = 8.8 Hz, 3H, CH_3C). ^{13}C NMR: δ 80.15 (C-11 and C-12), 51.41 (CH_3O), 31.27 (C-14), 29.33, 28.14, 29.08, 28.81, 28.37 (C-4 to C-9), 21.93 (C-15), 18.74 and 18.44 (C-10 and C-13), 13.63 (C-16). MS: *m/z* 270 (3%), 96 (100%).

Methyl 16-chloro-12,13-(ethoxycarbonylmethylene)-12-hexadecenoate (**10a**). Into a two-neck, round-bottomed flask, 0.51 g (1.7 mmol) of **8a** and 40 mg (0.63 matg) of activated Cu-bronze were placed under argon. The mixture was heated at 135°C for 15 min, and 0.350 mL (3.4 mmol) of ethyl diazoacetate was slowly added. After 15 min of stirring at this temperature, the mixture was cooled to room temperature, hexane was added and the crude mixture filtered through celite. Removal of solvent gave an oil that was submitted to bulb-to-bulb distillation at 120°C/20 Torr to remove ethyl maleate and ethyl fumarate. The resulting residue was purified by column chromatography with hexane/diethyl ether (9:1, vol/vol), affording 0.3 g (0.78 mmol, 46%) of **10a**. HRMS: Calcd. for $C_{21}H_{35}O_4Cl$: 386.222388. Found: 386.223434. IR: 2927, 2854, 1737, 1722, 1178 cm^{-1}. ^1H NMR (300 MHz) δ 4.10 (*q*, J = 6.8 Hz, 2H, CH_2O), 3.66 (*s*, 3H, CH_3O), 3.57 (*c*, 2H, CH_2Cl), 2.58 (*c*, 2H, $ClCCCH_2C{=}$), 2.40 (*tt*, J = 7.2 and 1.5 Hz, 2H, $CCCCH_2C{=}$), 2.29 (*t*, J = 7.4 Hz, 2H, CH_2CO), 2.04 (*s*, 1H, CH), 2.01 (*m*, J = 6.9 Hz, 2H, $ClCCH_2$), 1.57 (*c*, 4H, $CH_2CC{=}$ and CH_2CCO), 1.26 (*b*, 12H, $=CCC(CH_2)_6$), 1.23 (*t*, J = 7.2 Hz, 3H, CH_3C). ^{13}C NMR (50 MHz): δ 176.63 (CHCO), 174.21 (C-1), 107.38 (C-13), 103.98 (C-12), 59.82 (CH_2O), 51.34 (CH_3O), 44.00 (C-16), 34.01 (C-2), 29.83 (C-15), 29.37, 29.30, 29.18 and 29.05 (C-4 to C-9), 26.88 (C-10), 24.86 (C-3), 24.43 (C-11), 22.13 (CH), 21.83 (C-14), 14.33 (CH_3C). MS: *m/z* 386 (6%), 315 (100%), 201 (56%), 173 (50%).

Methyl 10,11-(ethoxycarbonylmethylene)-10-tetradecenoate (**11a**). The procedure described above afforded **11a** in 42% yield from **9a** (0.454 g, 1.9 mmol). HRMS: Calcd. for $C_{19}H_{32}O_4$: 324.230060. Found: 324.229995. IR: 2969, 2925, 1739, 1722, 1176 cm^{-1}. ^1H NMR (200 MHz): δ 4.10 (*q*, J = 7.2 Hz, 2H, CH_2O), 3.67 (*s*, 3H, CH_3O), 2.38 (*c*, 4H, $CH_2C{=}$), 2.29 (*t*, J = 7.6 Hz, 2H, CH_2CO), 2.02 (*s*, 1H, CH), 1.58 (*c*, 6H, $CH_2CC{=}$ and CH_2CCO), 1.29 (*b*, 10H, $=CCC(CH_2)_4$ and CH_2CH_3), 1.23 (*t*, J = 7.2 Hz, 3H, CH_3CH_2O), 0.95 (*t*, J = 7.4 Hz, 3H CH_3CH_2C). ^{13}C NMR (50 MHz): δ 177.05 (CHCO), 174.26 (C-1), 105.76 and 105.51 (C-10 and C-11), 59.72 (CH_2O), 51.42 (CH_3O), 34.06 (C-2), 29.15, 29.12 and 29.08 (C-4 to C-7), 26.92 (C-8) 26.51 (C-12), 24.89 (C-3), 24.47 (C-9), 22.24 (CH), 20.38 (C-13), 14.40 (CH_3CH_2O), 13.84 (C-14). MS: *m/z* 293 (10%), 251 (100%).

Methyl [2,2,3,3,-$^2H_4$]11,12-(ethoxycarbonylmethylene)-11-hexadecynoate (**18a**). The procedure applied to **17** (0.48 g, 3.57 mmol) gave **18a** in 83% yield. HRMS: Calcd. for $C_{21}H_{32}D_4O_4$: 356.286467. Found: 356.285461. IR: 2954, 2929, 2856, 1731, 1724, 1176 cm^{-1}. ^1H NMR (200 MHz): δ 4.08 (*q*, J = 7.2 Hz, 2H, CH_2O), 3.64 (*s*, 3H, CH_3O), 2.37 (*t*, J = 7.2 Hz, 4H, $CH_2C{=}$), 2.00 (*s*, 1H, CH), 1.51 (*c*, 4H, $CH_2CC{=}$), 1.29-1.39 (*c*, 12H, $=CC(CH_2)_5$ and CH_2CH_3), 1.21 (*t*, J = 6.2 Hz, 3H, CH_3CH_2O), 0.89 (*t*, J = 7.4 Hz, 3H, CH_3CH_2C). ^{13}C NMR (50 MHz): δ 177.02 (CHCO), 174.29 (C-1), 105.59 (C-11 and C-12), 59.70 (CH_2O), 51.36 (CH_3O), 29.30, 29.17, 29.03 and 28.82 (C-4 to C-8 and C-14), 26.93 (C-9), 24.45 (C-10), 24.17 (C-13), 22.29 and 22.24 (CH and C-15), 14.38 (CH_3CH_2O), 13.71 (C-16). MS: *m/z* 356 (7%), 297 (30%), 284 (100%).

16-Chloro-12,13-(carboxymethylene)-12-hexadecenoic acid (**10b**). A solution of 0.15 g (0.39 mmol) of **10a** in 2 mL of 20% KOH in 98% EtOH was stirred at room temperature under argon. Disappearance of the starting material was followed by TLC. After completion of the reaction (10–18 h), 130 mg (0.34 mmol) of pure **10b** was obtained by extraction with CH_2Cl_2. A small sample was purified by column chromatography and elution with CH_2Cl_2/MeOH (90:10, vol/vol) for characterization. HRMS: Calcd. for $C_{18}H_{29}O_4Cl$: 344.439888. Found: 344.439876. IR: 3500–2200, 2927, 2854, 1703, 1697, 1417, 1282, 1232 cm^{-1}. ^1H NMR (300 MHz): δ 3.59 (*c*, 2H, CH_2Cl), 2.61 (*c*, 2H, $ClCCCH_2C{=}$), 2.43 (*c*, 2H, $CCCCH_2C{=}$), 2.34 (*t*, J = 6.9 Hz, 2H, CH_2CO), 2.04 (*s*, 1H, CH), 2.03 (*m*, J = 6.9 Hz, 2H, $ClCCH_2$), 1.58 (*c*, 4H, $CH_2CC{=}$ and CH_2CCO), 1.26 (*b*, 12H, $=CCC(CH_2)_6$). ^{13}C NMR (50 MHz): δ 183.61 (CHCO), 180.30 (C-1), 107.06 (C-13), 103.49 (C-12), 44.04 (C-16), 34.11 (C-2), 29.77 (C-15), 29.16, 29.07, 28.94 (C-4 to C-9), 26.77 (C-10), 24.58 (C-3), 24.47 (C-11), 21.96 (CH), 21.88 (C-14).

10,11-(Carboxymethylene)-10-tetradecenoic acid (**11b**). Using the same hydrolysis and purification procedures gave **11b** in 70% yield from **11a** (0.600 g, 2.12 mmol). HRMS: Calcd. for $C_{16}H_{26}O_4$: 282.183110. Found: 282.180732. IR: 3600–2200, 2929, 2856, 1703, 1226 cm^{-1}.

^{1}H NMR (200 MHz): δ 10.41 (*b*, 2H, COOH), 2.38 (*c*, 4H, CH_2C=), 2.32 (*t*, *J* = 7.4 Hz, 2H, CH_2CO), 2.01 (*s*, 1H, CH), 1.57 (*m*, 6H, CH_2CC= and CH_2CCO), 1.29 (*b*, 8H, =CCC$(CH_2)_4$), 0.94 (*t*, *J* = 7.2 Hz, 3H, CH_3). ^{13}C NMR (50 MHz): δ 183.91 (CHCO), 180.16 (C-1), 105.39 and 105.13 (C-10 and C-11), 34.15, (C-2), 28.99 and 28.89 (C-4 to C-7), 26.79 (C-8), 26.47 (C-12), 24.61 (C-3), 24.41 (C-9), 22.04 (CH) 20.32 (C-13), 13.82 (C-14).

[2,2,3,3-$^2H_4$]11,12-(Carboxymethylene)-11-hexadecynoic acid (**18b**). Using the same hydrolysis procedure furnished **18b** in 86% yield from **18a** (528 mg, 1.47 mmol). HRMS: Calcd. for $C_{18}H_{26}D_4O_4$: 314.239517. Found: 314.237953. IR: 3000–2500, 2956, 2929, 2856, 1693, 1420, 1265, 740 cm^{-1}. ^{1}H NMR: δ 11.70 (*b*, 2H, COOH), 2.33 (*t*, *J* = 6.8 Hz, 4H, CH_2C=), 1.94 (*s*, 1H, CH), 1.55–1.05 (*c*, 16H, =CC$(CH_2)_6$ and C$H_2)_2CH_3$), 0.83 (*t*, *J* = 7.2 Hz, 3H, CH_3). ^{13}C NMR: δ 184.21 (CHCO), 180.61 (C-1), 105.24 (C-11 and C-12), 29.28, 29.18, 29.11, 28.948, 28.76 (C-4 to C-8 and C-14), 26.89 (C-9), 24.45 (C-10), 24.17 (C-13), 22.34 (C-15), 22.10 (CH), 13.74 (C-16).

Methyl 16-chloro-12,13-methylene-12-hexadecenoate (**12**). A modification of previously reported procedures (4,5) was followed. Diacid **10b** (100 mg, 0.29 mmol) was treated with three equivalents of $(COCl)_2$, and the mixture was stirred at room temperature for 1 h. Excess $(COCl)_2$ was removed with a stream of argon, and the residue thoroughly dried at 0.1 Torr for 2 h. After this time, dry CH_2Cl_2 (1 mL) and 0.3 mL (0.3 mmol) of a 1 M solution of $ZnCl_2$ in diethyl ether was added and the mixture stirred at room temperature for 10 min under argon. The resulting cyclopropenium ion solution was added, at −40°C under argon, to 0.6 mL of a 1 M solution of $NaBH_4$ (0.6 mmol) in 0.5 M NaOH in dry MeOH. Stirring was continued for 30 min at this temperature, and the suspension was warmed to room temperature and treated with 0.1 N HCl. Extraction with CH_2Cl_2 gave an oil that was purified by column chromatography using hexane to furnish 15 mg (0.05 mmol, 17%) of ester **12**. HRMS: Calcd. for $C_{18}H_{31}O_2Cl$: 314.530507. Found: 314.530456. IR: 2925, 2854, 1739, 1434, 1170, 1008 cm^{-1}. ^{1}H NMR (200 MHz): δ 3.66 (*s*, 3H, CH_3O), 3.56 (*t*, *J* = 6.6 Hz, 2H, CH_2Cl), 2.56 (*tt*, *J* = 7.0 and 1.3 Hz, 2H, $ClCCCH_2C$=), 2.38 (*tt*, *J* = 7.2 and 1.8 Hz, 2H, $CCCCH_2C$=), 2.31 (*t*, *J* = 7.6 Hz, 2H, CH_2CO), 2.03 (*m*, *J* = 6.8 Hz, 2H, $ClCCH_2$), 1.28 and 1.58 (*c*, 16H, =CC$(CH_2)_8$), 0.79 (*s*, 2H, ring CH_2). ^{13}C NMR (50 MHz): δ 174.33 (C-1), 110.94 (C-13), 107.61 (C-12), 51.43 (CH_3O), 44.48 (C-16), 34.09 (C-2), 30.39 (C-15), 29.47, 29.35, 29.23, 29.12 (C-4 to C9), 27.28 (C-10), 25.97 (C-11), 24.97 (C-3), 23.27 (C-14), 7.34 (C-17). MS: *m/z* 314 (1%), 283 (17%), 130 (18%), 95 (100%).

Methyl [17-H_2]12,13-methylene-12-hexadecenoate (**3a**). The same procedure was followed to prepare **3a** in 18% yield from **13a** (21 mg, 0.068 mmol). HRMS: Calcd. for $C_{18}H_{31}DO_2$: 281.246507. Found: 281.246629. IR: 2925, 2854, 1714 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.67 (*s*, 3H, CH_3O), 2.37 (*c*, 4H, CH_2C=), 2.30 (*t*, *J* = 7.5 Hz, 2H, CH_2CO), 1.58 (*c*, 6H, CH_2CC= and CH_2CCO), 1.28 (*b*, 12H, =CCC$(CH_2)_6$), 0.94 (*t*, *J* = 7.5 Hz, 3H, CH_3), 0.75 (*s*, 1H, CHD). ^{13}C NMR (75 MHz): δ 174.33 (C-1), 108.99 and 109.34 (C-12 and C-13), 51.44 (CH_3O), 34.11 (C-2), 29.14, 29.23, 29.37, 29.40, 29.51 (C-4 to C-9), 28.06 (C-14), 27.37 (C-10), 26.01 (C-11), 24.95 (C-3), 20.73 (C-15), 13.99 (C-16), 6.92 (*t*, *J* = 25 Hz, CHD). MS: *m/z* 281 (2%), 250 (7%), 82 (100%).

Methyl [17-^2H]11,12-methylene-11-hexadecenoate (**4a**). The same procedure was followed to prepare **4a** in 13% yield from **13b** (23 mg, 0.070 mmol). HRMS: Calcd. for $C_{18}H_{31}DO_2$: 281.246507. Found: 281.246254. IR: 2925, 2854, 1714 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.66 (*s*, 3H, CH_3O), 2.36, (*c*, 4H, CH_2C≡), 2.29 (*t*, *J* = 7.8 Hz, 2H, CH_2CO), 1.63–1.47 (*c*, 6H, CH_2CC≡ and CH_2CCO), 1.39–1.24 (*c*, 12H, ≡CCC$(CH_2)_5$ and CH_2CH_3), 0.9 (*t*, *J* = 7.5 Hz, 3H, CH_3C), 0.73 (*s*, 1H, CHD). ^{13}C NMR (75 MHz): δ 174.37 (C-1), 109.18 (C-11 and C-12), 51.42 (CH_3O), 34.12 (C-2), 29.52, 29.35, 29.31, 29.20, 29.11 (C-4 to C-8), 27.34 (C-14), 26.01 (C-9), 25.98 and 25.69 (C-10 and C-13), 24.93 (C-3), 22.42 (C-15), 13.83 (C-16), 6.98 (*t*, *J* = 25 Hz, CHD). MS: *m/z* 281 (5%), 250 (6%), 96 (100%).

Methyl [17-^2H]10,11-methylene-10-hexadecenoate (**5a**). The same procedure was followed to prepare **5a** in 15% yield from **13c** (20 mg, 0.068 mmol). HRMS: Calcd. for $C_{18}H_{31}DO_2$: 281.246507. Found: 281.246629. IR: 2925, 2854, 1714 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.65 (*s*, 3H, CH_3O), 2.35 (*c*, 4H, CH_2C=), 2.29 (*t*, *J* = 7.8 Hz, 2H, CH_2CO), 1.63–1.47 (*c*, 6H, CH_2CC= and CH_2CCO), 1.41–1.22 (*c*, 12H, =CCC$(CH_2)_4$ and C$H_2)_2CH_3$), 0.9 (*t*, *J* = 6.6 Hz, 3H, CH_3C), 0.73 (*s*, 1H, CHD). ^{13}C NMR (75 MHz): δ 174.23 (C-1), 109.33 and 109.22 (C-10 and C-11), 51.36 (CH_3O), 34.12 (C-2), 31.62 (C-14), 29.31, 29.20, 29.14 (C-4 to C-7), 27.36 and 27.08 (C-8 and C-13), 26.01 (C-9 and C-12), 24.96 (C-3), 22.45 (C-15), 14.01 (C-16), 6.99 (*t*, *J* = 25 Hz, CHD). MS: *m/z* 281 (1%), 250 (5%), 110 (26%), 69 (100%).

Methyl [15-^2H]10,11-methylene-10-tetradecenoate (**2a**). The same procedure was followed to prepare **2a** in 48% yield from **11b** (49 mg, 0.174 mmol). HRMS: Calcd. for $C_{16}H_{27}DO_2$: 253.215207. Found: 253.214897. IR: 2925, 2854, 1737, 1712 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.65 (*s*, 3H, CH_3O), 2.34 (*c*, 4H, CH_2C=), 2.29 (*t*, *J* = 7.5 Hz, 2H, CH_2CO), 1.56 (*c*, 6H, CH_2CC= and CH_2CCO), 1.26 (*b*, 8H, =CCC$(CH_2)_4$), 0.91 (*t*, *J* = 7.4 Hz, 3H, CH_3C), 0.73 (*s*, 1H, CHD). ^{13}C NMR (75 MHz): δ 174.33 (C-1), 109.32 and 109.07 (C-10 and C-11), 51.44 (CH_3O), 34.10 (C-2), 29.70, 29.29, 28.19 (C-4 to C-7), 28.06 (C-12), 27.33 (C-8), 25.99 (C-9), 24.94 (C-3), 20.72 (C-13), 13.97 (C-14), 6.92 (*t*, *J* = 25 Hz, CHD). MS: *m/z* 253 (18%), 222 (48%), 110 (50%), 69 (100%).

Methyl [2,2,3,3,17-$^2H_5$]11,12-methylene-11-hexadecenoate (**6a**). The same procedure was followed to prepare **6a** in 36% yield from **18b** (51 mg, 0.160 mmol). HRMS: Calcd. for $C_{18}H_{28}D_4O_2$: 285.925136. Found: 285.925666. IR: 2925, 2854, 1739, 1463, 1434, 1267, 1170 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.65 (*s*, 3H, CH_3O), 2.36 (*c*, 4H, CH_2C=), 1.51 (*c*, 4H, CH_2CC=), 1.41–1.20 (*c*, 12H, =CCC$(CH_2)_5$ and CH_2CH_3), 0.90 (*t*, *J* = 7.2 Hz, 3H, CH_3C), 0.73 (*s*, 1H, CHD). ^{13}C NMR (75 MHz): δ 174.33 (C-1), 109.22 (C-11 and C-12), 51.38 (CH_3O), 29.56, 29.34, 29.17, 29.01, 28.89, (C-4 to C-8), 27.37 (C-14), 26.01 (C-9), 25.72 (C-10 and C-13), 22.45 (C-15), 13.85 (C-16), 7.00 (*t*, *J* = 25 Hz, CHD).

Methyl [16-^2H]12,13-methylene-12-hexadecenoate (**1a**). A mixture of 5 mg (0.016 mmol) of **12**, 7 mg (0.017 mmol) of $NaBD_4$ and 0.3 mL of DMSO was stirred at 70°C for 24 h. After this time, extraction with hexane followed by column chromatography afforded 3 mg (0.011 mmol, 67%) of **1a**. HRMS: Calcd. for $C_{18}H_{31}DO_2$: 281.246507. Found: 281.246629. IR: 2925, 2854, 1714 cm^{-1}. ^{1}H NMR (300 MHz): δ 3.65 (*s*, 3H, CH_3O), 2.37 (*c*, 4H, CH_2C=), 2.30 (*t*, *J* = 7.8 Hz, 2H, CH_2CO), 1.57 (*c*, 6H, CH_2CC= and

CH_2CCO), 1.27 (*c*, 12H, $=CCC(CH_2)_6$), 0.90 (*c*, 2H, CH_2D), 0.73 (*s*, 1H, ring CH_2). ^{13}C NMR (75 MHz, DEPT): δ 174.32 (C-1), 109.50 and 109.16 (C-12 and C-13), 51.43 (CH_3O), 34.11 (C-2), 29.14, 29.23, 29.37, 29.40, 29.51 (C-4 to C-9), 28.05 (C-14), 27.37 (C-10), 26.01 (C-11), 24.95 (C-3), 20.63 (C-15), 14.10 (C-16), 7.33 (C-17).

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COMMUNICATION

Synthesis of Arachidonic Acid Metabolites by Syrian Hamster Platelets and Peritoneal Cells

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In this study, the metabolism of arachidonic acid by hamster platelets and peritoneal macrophages was assessed. Peritoneal macrophages stimulated *in vitro* with the calcium ionophore A23187 or stimulated *in vivo* by intraperitoneal injections of opsonized zymosan produced prostaglandin E_2, thromboxane B_2 (TxB_2) and 6-keto-prostaglandin $F_{1\alpha}$, as determined by radioimmuno assays. Leukotriene B_4 (LTB_4), and 11- and 15-hydroxyeicosatetraenoic acids (HETE), which were identified by reverse-phase high-performance liquid chromatography coupled with diode array detection, were produced by peritoneal cells stimulated *in vitro* with A23187 but were not found in the peritoneal exudate following *in vivo* stimulation with opsonized zymosan. Synthesis of 11- and 15-HETE, but not LTB_4, was inhibited by 1 μM indomethacin but not by 10 μM nordihydroguaiaretic acid, which did inhibit LTB_4 synthesis. Washed hamster platelets were prepared and shown to synthesize TxB_2, 12-HETE and 12-hydroxyheptadecatrienoic acid following stimulation with thrombin. This paper is the first to report on eicosanoid metabolism in tissues related to atherosclerosis, thrombosis and inflammation in hamsters.
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The Syrian hamster has become a model of choice for the study of the effects of dietary lipids on circulating plasma lipoprotein concentrations due to similarities in cholesterol metabolism in hamsters and humans (1-3). While the concentration of circulating lipids is an important risk factor for the development of cardiovascular disease (CVD), atherosclerosis is being recognized as a chronic inflammatory disease in arteries, which is similar to inflammatory events in other tissues (4,5).

Platelets, neutrophils, monocytes, macrophages and smooth muscle cells produce cytokines and other inflammatory mediators involved in the progression of atherosclerotic lesions (4). One class of inflammatory compounds susceptible to dietary or pharmaceutical modulation are the products of the arachidonic acid (AA) cascade. Prostaglandins, which are products of the cyclooxygenase (CO) pathway, are largely immunomodulatory and directly affect platelet function (6). The 5-lipoxygenase product leukotriene B_4 (LTB_4) is one of the most potent chemotactic agents (7); leukotrienes C_4, D_4 and E_4 (LTC_4, LTD_4 and LTE_4) induce smooth muscle cell contraction and can increase post-capillary venule permeability (8,9); and hydroxyeicosatetraenoic acids (HETE) have been implicated in the regulation of several cellular capacities such as phospholipase A_2 activity (10) and calcium mobilization (11).

Dietary lipids can have a profound effect on tissue AA levels and eicosanoid production (12-14). Consequently, the basis for dietary lipid-induced changes in CVD risk may be considered to be a function of changes in the capacity for lipid mediator synthesis in addition to changes in circulating plasma lipid levels (15,16). In order to more thoroughly characterize the hamster as a model for the evaluation of CVD risk, changes in potential risk factors other than plasma lipid levels must be examined, especially when considering the effects of dietary lipids of the n-3 class whose antiatherogenic effects have been linked to altered eicosanoid production (15,16). However, no information exists on AA metabolism in hamster cells. Therefore, in this study, platelets and macrophages were prepared from Syrian hamsters in order to characterize their formation of CO and lipoxygenase (LO) products.

MATERIALS AND METHODS

Materials. AA metabolites were purchased from Cayman Chemical Co. (Ann Arbor, MI). Male Syrian hamsters (90-110 g) were purchased from Charles River Laboratories (Wilmington, MA).

Stimulation of peritoneal cells in vitro. Peritoneal cells were harvested by washing the peritoneal cavity twice with 5 mL of physiologically buffered saline (PBS) and were washed twice by centrifugation. The peritoneal exudate population consisted mainly of macrophages (90%) as determined by standard morphological criteria using cytospin preparations stained with May-Grünwald-Giesma stain. Pooled cell suspensions (10^6 cells per mL) were incubated in RPMI medium (Hazelton Biologics Inc., Lenexa, KS) in 3-mL aliquots for one hour at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The adherent cells (> 98% macrophages) were washed and stimulated for 15 min at 37°C with 5 μM calcium ionophore A23187, and the culture media were collected for eicosanoid analysis. In inhibition studies, the adherent cells were preincubated for 15 min with either 1 μM indomethacin or 10 μM nordihydroguaiaretic acid (NDGA) prior to stimulation with A23187 as above.

Eicosanoid production in vivo. Opsonized zymosan was prepared as previously described (17). Opsonized zymosan (2 mg in 1 mL saline) was injected intraperitoneally (18), and after 30 min the animals were killed and the peritoneal

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Abbreviations: AA, arachidonic acid; CO, cyclooxygenase; CVD, cardiovascular disease; HETE, hydroxyeicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4 leukotriene E_4; LO, lipoxygenase; NDGA, nordihydroguaiaretic acid; PBS, physiologically buffered saline; PGB_1, prostaglandin B_1; PGE_2, prostaglandin E_2; PRP, platelet-rich plasma; RIA, radioimmunoassay; TxB_2, thromboxane B_2; UV, ultraviolet.

cavity was washed twice with 5 mL of PBS. The peritoneal wash was centrifuged (700 × *g* for 10 min at 4°C), and the supernatant was removed for eicosanoid analysis.

Preparation and stimulation of washed platelets. Platelet-rich plasma (PRP) was prepared by centrifugation (650 × *g*, 10 min) of citrated blood (0.38% citrate) collected from hamsters anesthetized with metafane. The centrifugation was repeated two more times, and the PRP was pooled and acidified with citric acid (pH 6.4). The PRP was then centrifuged (900 × *g*, 10 min), and the pellet was washed once with calcium-free modified tyrode's buffer (19). Aggregation studies were carried out using a Payton aggregometer (Payton Instruments, Buffalo, NY) with 6 × 10^5 platelets per μL at 37°C. Platelets were preincubated at 37°C for 15 min, and $CaCl_2$ (0.1 mM) was added to the suspension before the addition of agonist. Platelets were stimulated with thrombin (0.4 U/mL), which was the concentration that caused 75% platelet aggregation, and the reaction was stopped after 4 min by the addition of acetic acid (0.4% final) prior to eicosanoid analysis.

Sample extraction and analysis. Samples were extracted and analyzed for eicosanoids by radioimmunoassay (RIA) or reverse-phase high-performance liquid chromatography (RP-HPLC) as previously described (13). Separation by RP-HPLC of compounds produced by peritoneal cells was accomplished using a methanol/water (68:32, vol/vol) mobile phase, and for platelet products using methanol/water (72:28, vol/vol).

RESULTS

Eicosanoid production by peritoneal cells. Three peaks were identified in HPLC of the supernatant of A23187-stimulated peritoneal cells that had the characteristic ultraviolet (UV) absorption spectra of known LO products (Fig. 1). Compound C1 co-eluted with LTB_4 and possessed a UV absorption spectrum identical to that of authentic LTB_4, with a λ max at 270 nm (Fig. 2). The identity of LTB_4 was further confirmed by enzyme-linked immunosorbant assay (Cayman Chemical Co., Ann Arbor, MI), which shows little cross-reactivity (0.3%) with isomers of LTB_4 (data not shown). Compounds C2 and C3 co-eluted with 15- and 11-HETE, respectively, and possessed UV absorption spectra (Fig. 3) characteristic of conjugated dienes with distinctively different λ max at 236 nm and 234.5 nm corresponding to the λ maxima of 15-HETE and 11-HETE, respectively (20). No peaks co-

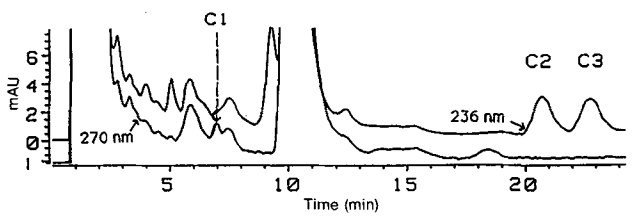

FIG. 1. Reversed-phase high-performance liquid chromatography (RP-HPLC) of supernatant of hamster peritoneal cells stimulated with calcium ionophore A23187. Adherent hamster peritoneal cells were stimulated with 5 μM A23187 for 15 min at 37°C. Separation was accomplished by RP-HPLC on a Whatman (Maidstone, England) Partisphere C-18 column (6 mm × 12.5 cm) using a methanol/water (68:32, vol/vol) mobile phase containing 5 mM ammonium acetate and 1 mM ethylenediaminetetraacetic acid, pH 5.6 and a flow rate of 0.9 mL/min. Absorbance of the eluate was monitored at 270 nm and 236 nm.

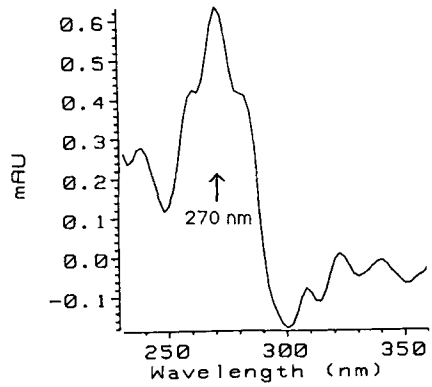

FIG. 2. Ultraviolet absorption spectrum (λ max 270 nm) of compound 1 (see Fig. 1) produced by peritoneal cells stimulated with 5 μM calcium ionophore A23187 for 15 min at 37°C.

eluting with LTB_4, C_4, D_4 or E_4, nor with 5-, 8-, 11-, 12- or 15-HETE were observed following analysis of the peritoneal exudate by RP-HPLC after *in vivo* stimulation with opsonized zymosan (data not shown).

Cells stimulated *in vitro* with A23187 or *in vivo* with opsonized zymosan produced prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) (the stable metabolite of prostacyclin) and thromboxane B_2 (TxB_2) (the stable metabolite of thromboxane A_2) as determined by RIA (Table 1).

Incubation of cells with 1 μM indomethacin before stimulation with ionophore resulted in a 70% decrease in HETE production, but did not affect LTB_4 production compared to untreated cells (Table 2). Incubation of cells with 10 μM NDGA prior to stimulation with ionophore only resulted in a 27% decrease in HETE synthesis but decreased LTB_4 synthesis to levels below limits of detection.

Eicosanoid production by washed platelets. Three AA metabolites were identified in extracts of stimulated platelets; TxB_2, 120 ± 10 ng per 10^9 platelets; 12-hydroxyheptadecatrienoic acid (HHT), 85 ± 15 ng per 10^9 platelets; and 12-HETE , 70 ± 14 ng per 10^9 platelets (means ± SEM, n = 5). HHT and 12-HETE were identified by HPLC by comparing their retention times to those of

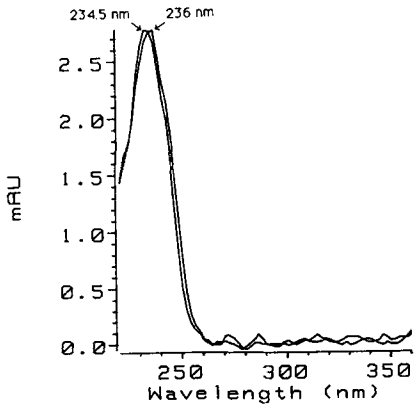

FIG. 3. Ultraviolet absorption spectra of compounds 2 (λ max 236) and 3 (λ max 234.5) (see Fig. 1) produced by peritoneal cells stimulated with 5 μM calcium ionophore A23187 for 15 min at 37°C.

TABLE 1

Synthesis of Eicosanoids by Hamster Peritoneal Cells Stimulated *in vitro* with the Ionophore A23187 or *in vivo* by Peritoneal Injections of Opsonized Zymosan[a]

Stimulus	Eicosanoids (ng/10^6 cells)					
	PGE_2	6-k-$PGF_{1\alpha}$	TxB_2	LTB_4	11-HETE	15-HETE
A23187	0.25 ± 0.09	11.6 ± 4.7	0.39 ± 0.09	5.5 ± 1.3	14.1 ± 3.0	12.4 ± 2.8
Zymosan	2.01 ± 0.3	141 ± 45	0.75 ± 0.23	ND[b]	ND	ND

[a]Adherent hamster peritoneal cells were stimulated *in vitro* for 15 min with 5 μM A23187 prior to extraction and analysis of arachidonic acid metabolites. Peritoneal cells were stimulated *in vivo* by intraperitoneal injections of 2 mg opsonized zymosan for 30 min (means ± SD, n = 8). $P6E_2$, prostaglandin E_2; 6-k-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; TxB_2, thromboxane B_2; LTB_4, leukotriene B_4; HETE, hydroxyeicosatetraenoic. [b]ND, not detected.

TABLE 2

Effect of Inhibitors on Calcium Ionophore-Induced Production of LTB_4, 15-HETE and 11-HETE by Peritoneal Cells[a]

Treatment	ng per million cells		
	11-HETE	15-HETE	LTB_4
Control	11.5 ± 2.0	9.8 ± 1.4	5.0 ± 0.3
Indomethacin	3.3[b] ± 0.4	3.7[b] ± 0.2	4.9 ± 0.4
NDGA	8.1 ± 1.0	7.4 ± 0.9	ND[b,c]

[a]Peritoneal cells were pre-incubated without inhibitors (control), with indomethacin (1 μM) or with NDGA (10 μM) for 15 min prior to stimulation with 5 μM A23187 for 15 min. Means ± SD (n = 3). Abbreviations: LTB_4, leukotriene B_4; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid.
[b]Significantly different from control, $P < 0.01$.
[c]ND, not detected.

authentic standards and by their characteristic UV absorption spectra (data not shown).

DISCUSSION

The hamster has been a valuable model for understanding the metabolism of circulating lipoproteins and their regulation by factors such as dietary lipids (1–3,21). However, little has been reported on the production of AA metabolites in hamster even though these compounds are believed to be involved in the development of atherosclerosis, and their synthesis is susceptible to dietary as well as pharmaceutical modulation.

The hamster peritoneal cells examined in this study, like those of the rat, mouse and guinea pig, consist mostly of macrophages (> 90%). However, the AA metabolites produced in response to stimuli by this cell population differed from those produced in similar cell populations from other species (17,22–25). Stimulation of the adherent peritoneal cell population with the ionophore, A23187 resulted in the detection by RP-HPLC of three AA metabolites, LTB_4, 11-HETE and 15-HETE. In comparison, mouse peritoneal cells stimulated *in vitro* have been reported to produce LTB_4, LTC_4, LTE_4 and 5-, 12- or 15-HETE (25–27) and rat cells LTB_4, C_4 and D_4 (23). Adherent hamster peritoneal macrophages, like mouse peritoneal macrophages, also produced the CO-derived PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2, following ionophore stimulation as determined by RIA.

The treatment of cells with the CO inhibitor indomethacin, at concentrations which do not affect LO activity (28), as expected did not affect LTB_4 production, but resulted in a 70% decrease in HETE synthesis. Cells treated with the nonspecific LO inhibitor NDGA inhibited the synthesis of LTB_4, but not of 11- and 15-HETE. This suggests that the 11- and 15-HETE are synthesized *via* the CO pathway as has been previously reported in human umbilical arteries (29) and endothelial cells (30), fetal calf aorta (31) and cultured rat aorta smooth muscle cells (32).

In vivo stimulation of hamster peritoneal cells with opsonized zymosan resulted in the synthesis of greater amounts of PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 than were observed in cells stimulated *in vitro* (Table 1). This may be due to the potential contribution of other cells in the peritoneal cavity to the synthesis of prostaglandins. However, the production of hydroxy fatty acids or leukotrienes by as many as 1.5×10^7 cells was not detected following *in vivo* stimulation. In contrast, the mouse peritoneum subjected to the same stimulus produces in addition to the prostanoids, the cysteinyl-leukotrienes LTC_4 and LTE_4 in concentrations of 16 and 78 ng per 10^6 cells, respectively (17). Thus, the profile of AA-derived compounds produced by peritoneal cells shows species variations and, as with mouse peritoneal cells (22), is stimulus-dependent.

Washed hamster platelets produced the same major AA metabolites as platelets from other species, including humans, following stimulation with agonist (33). 12-HETE, a product of the 12-LO pathway, and the CO-derived HHT as well as TxB_2, were identified in supernatants of platelets stimulated with thrombin. The preparation of platelets for the determination of aggregability and eicosanoid production could be of value in the determination of the potential anti-atherogenic or anti-thrombotic effects of drugs or special diets in this species.

Differences in eicosanoid metabolism between different species underscore the need to determine AA metabolites produced in animal models used for the study of lipid metabolism and its relationship to diseases such as atherosclerosis. The identification, in this study, of AA metabolites synthesized by hamster cells should enable, in the future, a more thorough evaluation of the potential anti-atherogenic effects of dietary or pharmaceutical interventions in this species.

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